Investigation of Apoptosis by Conditional Gene Expression.

A Thesis Submitted in Satisfaction of the Requirements for the Degree of Doctor of Philosophy

by

Roger D. G. Malcomson

in the

University of Edinburgh.

1996

Declaration.

This thesis has been composed entirely by myself and details work carried out by myself unless specifically stated otherwise in the text.

R. D. G. Malcomson

Edinburgh,

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THE UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

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poptosis is a form of cell death that occurs in individual cells in normal and diseased tissues. It occurs as a nsequence of activation of a program of molecular events that results in the dismantlement and clearance of lls, often without induction of an inflammatory response. Many of the molecular components that regulate and ecute apoptosis have been identified. Some of these regulators are, or are related to, genes that are altered in oplasia, such as c-myc,p53 and Bcl-2. The effector molecules belong to a class of cysteine proteases, known the ICE-like proteases (e.g. Nedd2). Whilst the known components can have identifiable activity in apoptosis d (or) cell cycle control, it has been suggested that they interact to control apoptosis. Further, it is not known w the regulatory molecules interact upon downstream effectors to control apoptosis induction. Until recently, has not been possible to examine the role of apoptosis genes in combination other than by combinatorial mouse ockouts - a very time-consuming and expensive exercise. It was therefore decided to direct the expression of veral apoptosis-related genes (namely c-*myc*, p53, p21WAF/CIP, and Nedd2) in tissue culture in order to ovide useful tools to answer questions about the role of such genes in the control of apoptosis.

wing to the limitations of existing conventional expression technology, where test genes are constitutively verexpressed from a strong viral promoter often in transient expression assays, use was made of conditional inigenes: 1) A temperature-sensitive (ts) murine p53 (p53val135) was employed to investigate the sensitivity of tivated c-Ha-*ras*-transformed rat embryo fibroblasts (Clone 6) to DNA-damage induced by the genotoxic emotherapeutic drugs etoposide and bleomycin; 2) An oestrogen-regulable c-*myc*-oestrogen receptor hormone nding domain fusion protein (*myc*-ER) was used in conjunction with p53val135 in order to investigate whether rced expression of phenotypically wild-type p53 was sufficient to trigger apoptosis by c-*myc* in Clone 6 and at-1 fibroblasts; and 3) Vectors were constructed that contain apoptosis genes under the control of semi-nthetic promoters based upon the inducible *E. coli lac* operator-repressor system or a promoter containing ast Gal-4 binding sites inducible by a tamoxifen-sensitive VP16GaER^{tm} chimaeric *trans*-activator protein.

esults showed that: 1) Expression of ts p53 at the permissive temperature protected Clone 6 cells from totoxic drug-induced apoptosis, probably by enforcing a cell cycle arrest in G1. 2) Co-expression of myc-ER d ts p53 yielded no stable cell lines probably due to biologically significant basal activation of both p53val135 d myc-ER under the culture conditions used. This is consistent with a co-operative role for c-myc and p53 in poptosis triggering. 3) Control of expression in Rat-1 cells of the ICE-like protease Nedd2 (the mouse mologue of human ICH-1) by the VP16GalER^{tm} system was tight enough to allow development of stably-ansfected cells, which upon induction with 4-hydroxytamoxifen, rapidly underwent apoptosis. In contrast, ansfections with a LacI-repressible Nedd2 expression vector could not produce repressed stable expression vels that were low enough to be compatible with colony survival following selection in tissue culture.

this work, conditional expression technology was applied to the problem of control of apoptosis and shows at gene expression experiments that increase the susceptibility of cells to apoptosis can be carried out in a gulated fashion. Using this approach, a cytoprotective role of wild type p53-mediated growth arrest was iscovered that was abrogated by c-*myc*. In addition, cell lines were developed that are suitable for the iochemical characterisation of the action of Nedd2 protease.

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Table of Contents.

Abbreviations.

ac	acetyl
actD	actinomycin D
AmpR	ampicillin resistance
AT	ataxia telangiectasia
ATP	adenosine triphosphate
BBS	buffered BES
BES	*N,N-bis* (2-hydroxyethyl)-2-aminoethanesulphonic acid
BH	Bcl-2 homology domain
BSA	bovine serum albumin
CD	cluster of differentiation
CDK	cyclin-dependent kinase
ced-3	C. Elegans cell death gene 3
CHO	ketone
CHX	cyclohexamide
CMK	chloromethyl ketone
CTL	cytotoxic T lymphocytes
DBD	DNA-binding domain
DDW	doubly-distilled water
DEPC	diethyl pyrocarbonate
DEVD	Asp-Glu-Val-Asp
DEX	dexamethasone
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
EBV	Epstein-Barr virus
EDTA	diaminoethane tetra-acetic acid
ER	oestrogen receptor

ER^{tm}	oestrogen receptor (tamoxifen mutant)
FCS	foetal calf serum
FITC	fluoroscein iso-thiocyanate
G418	Geneticin
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HBD	hormone binding domain
hER	human oestrogen receptor
HIFCS	heat-inactivated foetal calf serum
HINCS	heat-inactivated neonatal calf serum
hMT	human metallothionein
Hsp (or HSP)	heat shock protein
HygR	hygromycin B resistance
ICE	Interleukin-1β converting enzyme
Ig	immunoglobulin
IGF	Insulin-like growth factor
IGF-BP	IGF-binding protein
IL-1β	Interleukin-1β
IL-1β	Interleukin-1β
INF-γ	Interferon-γ
IPTG	isopropylthio-β-D-galactopyranoside
kDa	kilodalton
*lac*OP	*lac* operator sites
*lac*Z	*E. coli* β-galactosidase gene
LB	Luria-Bertani medium
LTR	long terminal repeat
MCS	multiple cloning site
mER	mouse oestrogen receptor
mMT	mouse metallothionein
MoMSV	Moloney murine sarcoma virus
MOPS	3-[N-morpholino]propane sulphonic acid

MRE	metal response element
mRNA	messenger RNA
MSV	murine sarcoma virus
MTF	MRE-binding transcription factor
Nedd2	NPC expressed developmentally downregulated gene 2
neo	Tn5 neomycin (kanamycin and G418) resistance gene
NK	natural killer
NLS	nuclear localisation signal
OD	optical density
ORF	open reading frame
pA (or polyA)	polyadenylation signal
PAC	puromycin N-acetyl transferase
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PIP_2	phosphoinositol bisphosphate
PKC	protein kinase C
PMA	phorbol myristate acetate
PNK	polynucleotide kinase
QARCG	Gln-Ala-Arg-Cys-Gly
RNA	ribonucleic acid
RSV	Rous sarcoma virus
RT-PCR	reverse transcription polymerase chain reaction
rtTA	reverse tetracycline transactivator
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
SIN	self-inactivating (retrovirus)
SSC	saline sodium citrate buffer
SSPE	saline phosphate EDTA buffer
SV40	Simian virus 40

TAE	Tris-acetate/EDTA electrophoresis buffer
TBE	Tris-borate/EDTA electrophoresis buffer
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
tTA	tetracycline transactivator
YVAD	Tyr-Val-Ala-Asp

Chapter 1.

1. General Introduction

1.1 Introduction: Apoptosis

1.1.1 Definition

Apoptosis is a term applied to a dying cell which undergoes a defined series of morphological changes that results in the fragmentation of the cell and its components (Arends and Wyllie, 1991; Wyllie *et al*, 1980; Kerr *et al*, 1972) without the elicitation of an inflammatory response or interruption of organ function (Duvall and Wyllie, 1986; Wyllie *et al*, 1980). If the cell formed part of a tissue, the fragments are phagocytosed by tissue macrophages or adjacent cells (Savill *et al*, 1993; Wyllie *et al*, 1980).

Since the definition of an apoptotic cell is a morphological one, a consideration of the structural changes that occur during apoptosis is necessary.

1.1.2 Morphology (Fig. 1.1)

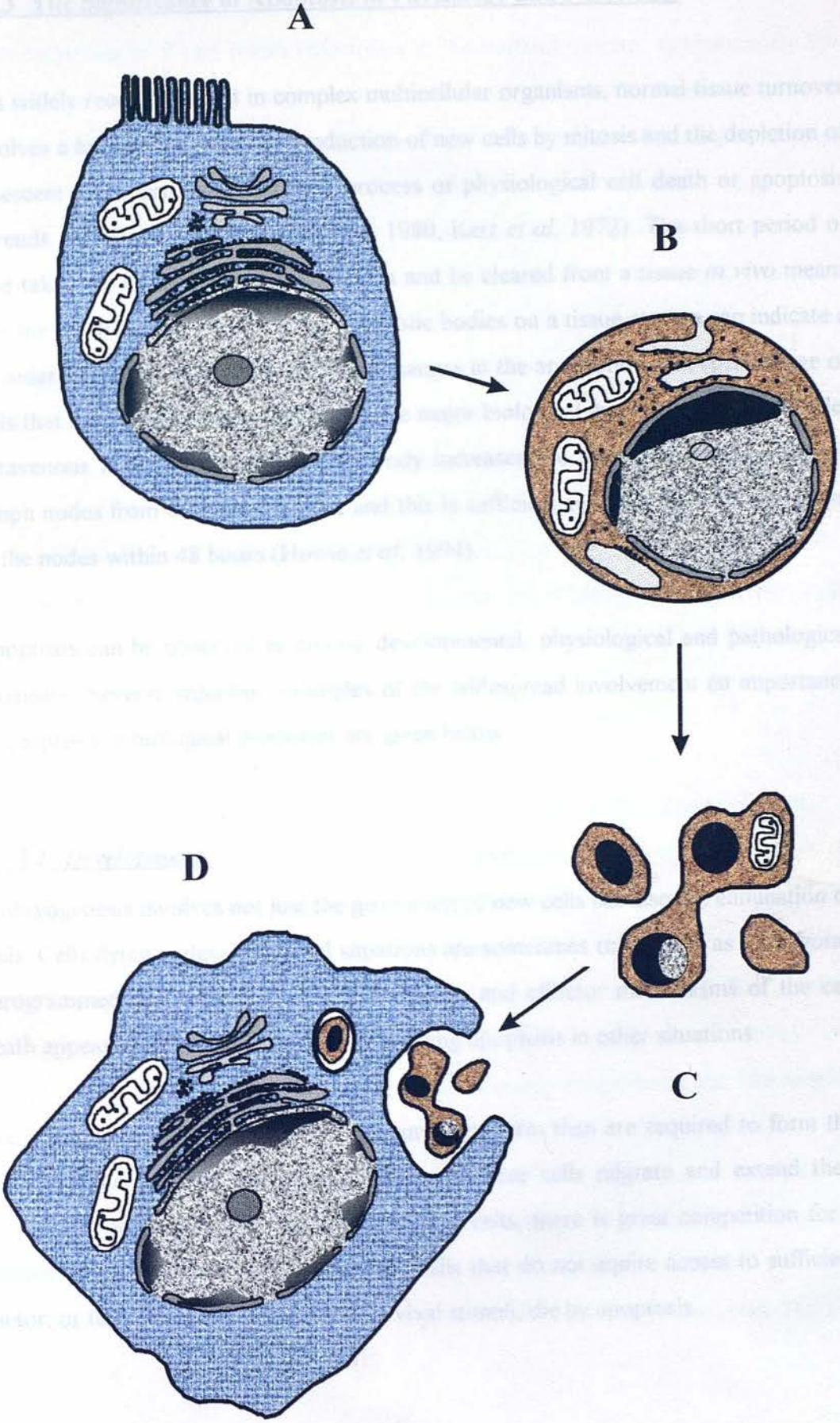
Apoptosis usually occurs in scattered cells in contrast to cells dying by necrosis which usually affects sheets of contiguous cells. The course of apoptosis of cells in tissues is of short duration (a few minutes in length) and can be divided into three main morphological phases (Arends and Wyllie, 1991; Wyllie *et al*, 1980). In the initial stage the cell loses contact with surrounding cells, the cytoplasm condenses and membrane specialisations (such as microvilli and desmosomes) disappear. The cell

surface becomes pock-marked as vesicles of endoplasmic reticulum fuse with the plasma membrane. Cytoskeletal elements aggregate into parallel arrays and ribosomes form semi-crystalline clumps. Organelles become closely packed but retain their integrity, although the endoplasmic reticulum becomes dilated. These changes are in contrast with the "high amplitude swelling" of mitochondria observed in cells undergoing necrosis (Trump *et al*, 1981). In addition to the cytoplasmic changes at this stage, nuclear chromatin becomes condensed and forms cresentic caps adjacent to the nuclear membrane (margination). The nucleolus usually disintegrates with scattering of the transcriptional complexes leaving behind the argyrophillic centre (Arends *et al*, 1990).

The second stage is characterised by extensive blebbing of the cytoplasm and nucleus and results in cellular fragmentation. Cellular fragments (apoptotic bodies) of variable size, characteristically consisting of densely-staining nuclear remnants set in markedly eosinophilic cytoplasm, are usually to be found in tissue sections as clusters of smooth, round, membrane-bound particles. These particles are usually phagocytosed by adjacent cells or shed into epithelial lumina. The majority of apoptotic bodies seen in tissue sections are contained within phagosomes as they remain visible for a few hours after engulfment (Bursch *et al*, 1990).

Degradation of apoptotic bodies occurs in the final stage. For apoptotic bodies contained within phagosomes or released into lumina or tissue culture medium, apoptosis culminates in membrane rupture and increased permeability to vital dyes (such as trypan blue or nigrosine). The morphology of apoptotic cells in this stage is similar to cell undergoing necrosis and is sometimes referred to as "secondary necrosis". There is, except in extreme instances, no inflammatory response or scar formation in tissues where apoptosis is occurring.

Fig. 1.1: **Morphological Features of Apoptotic Events.** A) A normal cell displaying a nucleus with nucleosome and chromatin; membrane specialisations, mitochondria, endoplasmic reticulum, ribosomes and Golgi apparatus. B) Early apoptotic cell which has shrunk in size, lost its membrane specialisations and contains dilated endoplasmic reticulum which is continuous with the cell surface. Also represented are intact mitochondria and nuclei with the beginnings of condensed chromatin that is seen to marginate at the nuclear membrane. C). Break-up of apoptotic cells into apoptotic bodies which may contain nuclear fragments with condensed chromatin. Mitochondria are still intact. D) Clearance of apoptotic bodies by phagocytosis and digestion by surrounding cells.


A
B
C
D

1.1.3 The Significance of Apoptosis in Physiology and Pathology.

It is widely recognised that in complex multicellular organisms, normal tissue turnover involves a balance between the production of new cells by mitosis and the depletion of senescent or superfluous cells by a process of physiological cell death or apoptosis (Arends and Wyllie, 1991; Wyllie *et al*, 1980; Kerr *et al*, 1972). The short period of time taken for an apoptotic body to form and be cleared from a tissue *in vivo* means that the identification of only a few apoptotic bodies on a tissue section can indicate a considerable cumulative cell loss. Small changes in the apoptotic index (percentage of cells that are apoptotic) can therefore have major biological importance. For example, intravenous injection of anti-CD4 antibody increases the apoptotic index in murine lymph nodes from 0.06% to 1.33%, and this is sufficient to halve the total cell count of the nodes within 48 hours (Howie *et al*, 1994).

Apoptosis can be observed in diverse developmental, physiological and pathological situations. Several important examples of the widespread involvement an importance of apoptosis in biological processes are given below.

1.1.3.1 Development.

Embryogenesis involves not just the generation of new cells but also the elimination of cells. Cells dying in developmental situations are sometimes referred to as undergoing "programmed cell death", but the morphology and effector mechanisms of the cell death appear to be identical to cells undergoing apoptosis in other situations.

Many more cells are produced in the neural ectoderm than are required to form the nervous system of the developing foetus. As these cells migrate and extend their axonal and dendritic processes towards target cells, there is great competition for a limited supply of neural survival factors. Cells that do not aquire access to sufficient factor, or to the incorrect mixture of survival stimuli, die by apoptosis.

1.1.3.2 Immunity.

In the selection of T and B cell repertoires in the immune system, approximately 95-97% of cells are eliminated by apoptosis (Deenen *et al,* 1990). These are the cells that produce self-reactive receptor specificities or do not undergo valid antigen receptor gene rearrangement. Apoptosis is also the main form of cell death triggered by cytotoxic T lymphocytes (CTL), natural killer (NK) cells and antibody-dependent cell mediated cytotoxic mechanisms (Cohen, 1993). Disruption of the normal control of lymphocyte repertoire control leads to autoimmunity or immune deficiency. Mice harbouring genetic defects in the *lpr* or *gld* loci (Fas or Fas ligand genes) develop an autoimmune syndrome resembling human systemic lupus erythematosus (SLE) (WatanabeFukunaga *et al,* 1992a) owing to a defect in deletion of autoreactive T cells. Conversely, HIV infection causes a progressive depletion of CD4+ T cells as a result of inappropriate apoptosis triggered by binding of viral envelope bound gp120-gp41 complex to the CD4 D1 domain (Gougeon and Montagnier, 1993; Ameisen, 1992).

1.1.3.3 Inflammation

At the onset of acute inflammation or infection, the inflammatory response is characterised by migration of large numbers of neutrophils and these are followed by eosinophils and monocytes. Neutrophils, eosinophils and monocytes die by apoptosis within a relatively short period. However death can be significantly delayed by proinflammatory cytokines such as C5a (neutrophils), IL-1β, TNF-α, INF-γ (monocytes) and IL-5 (eosinophils) (Stern *et al,* 1992; Mangan *et al,* 1991). The granules of these inflammatory cell types contain many components that can amplify the damage or the response to the damage at the site of inflammation. Therefore the control of the inflammatory reaction must take place in such a way as to limit the spillage of inflammatory cell contents. Apoptotic cells are normally rapidly engulfed by other cells, including macrophages and defects in the mechanism of such clearance may underlie some chronic inflammatory diseases. Mice without functional TGFβ1,

which accelerates eosinophil apoptosis, suffer from multifocal inflammatory disease and tissue necrosis (Shull *et al,* 1992).

1.1.3.4 Neoplasia.

In addition to areas of ischaemic necrosis, apoptosis occurs in actively growing and regressing tumours (Moore and Evan, 1987; Kerr *et al,* 1972) and influences the rate of tumour expansion (Wyllie, 1985). The slow growth of basal cell carcinoma of the skin despite high rates of mitosis is attributable to apoptosis (Kerr and Searle, 1972). Equally, suppression of cell death, for example by constitutive expression of Bcl2, is responsible for follicular lymphoma in humans (Tsujimoto *et al,* 1985) and the induction of hyperplastic and preneoplastic foci in experimental rodent hepatocarcinigenesis (Bursch *et al,* 1984). Suppression of cell death allows survival of damaged cells that would otherwise be deleted and therefore may contribute to tumour progression and metastasis (Harrison *et al,* 1995).

Cytotoxic drugs (Sen and d'Incalci, 1992) and moderate-dose irradiation can induce apoptosis (Wyllie *et al,* 1980; Searle *et al,* 1975). Tissues and tumours that have high proliferative activity have high rates of apoptosis and can be most susceptible to induction of apoptosis by these agents (Wyllie *et al,* 1980). However many cancer cell types are resistant to the normal induction of apoptosis and failure to undergo apoptosis in response to cytotoxic injury induced by chemotherapeutic agents may explain the clinical observation of drug resistance even when the pharmacological mechanisms of tumour-drug interactions appear to be operating effectively (Hickman, 1992).

1.1.4 Cell Biology of Apoptotic Cells.

1.1.4.1 Cytoplasmic Events.

The rapid increase in cell density at the onset of apoptosis is due to the voiding of water and ions, probably by channelling though the endoplasmic reticulum to the cell

surface (Arends and Wyllie, 1991). The mechanism by which ions are transported are not well understood, but it may involve extrusion of K^+ ions (Barbiero *et al*, 1995). It is interesting that a protein known to be upregulated in thymocyte apoptosis, RP-2, has similarity to a ligand-gated ion channel (Brake *et al*, 1994).

The cell size and shape changes during apoptosis require that dramatic changes to the cytoskeletal structure take place. Accordingly, although actin polymerisation is required for membrane blebbing and budding (Cotter *et al*, 1992), F-actin is depolymerised and specifically proteolysed in apoptosis (Mashima *et al*, 1995; Endresen *et al*, 1995). In some cell types, including hepatocytes, tissue transglutaminase non-specifically crosslinks protein into an insoluble shell that may stabilise the membrane blebs (Fesus *et al*, 1987).

Although increased transcription of certain specific mRNAs is required for the apoptosis of certain cell types (see below), there is evidence that the degradation of ribosomal RNA (rRNA) and mRNA late in the course of events is due, at least in part, to a site-specific endogenous ribonuclease (Houge *et al*, 1995; Delic *et al*, 1993). Specific cleavage of macromolecules is a characteristic feature of apoptosis as site-specific endonucleases and proteases (discussed in more detail below) are also important effector molecules in apoptosis.

1.1.4.2 Nuclear Events

In contrast to mitosis, the nuclear membrane remains intact in apoptosis and serves to contain condensed chromatin inside nuclear fragments. However, the nuclear lamina, to which chromatin is anchored, is disassembled by phosphorylation and subsequent depolymerisation of lamin filaments. Unlike mitosis where cdc2 kinase is responsible, the phosphorylation of lamins occurs in the absence of increased cdc2 kinase activity and is followed by degradation of the lamin monomers making the destruction of the nuclear lamina irreversible (Oberhammer *et al*, 1994; Lazebnik *et al*, 1993).

Change in chromatin structure is the most striking feature of apoptotic cells. While mitochondrial DNA remains intact in apoptosis (Topper and Studzinski, 1992; Murgia *et al,* 1992), chromatin is at first cleaved into large fragments of between 50 and 300kbp (observable by pulsed-field gel electrophoretic techniques) and it is thought that these fragments represent chromatin domains, the large loops of DNA that are attached to the nuclear matrix (Huang *et al,* 1995; Cain *et al,* 1994). The condensed chromatin that marginates to edge of the nucleus probably represents these large DNA fragments released from the constraints of the nuclear matrix (Oberhammer *et al,* 1993a; Oberhammer *et al,* 1993b). Subsequently, in most cell types, chromatin is further cleaved into much smaller fragments that are multiples of 180-200bp. These fragments are caused by double strand cleavage of DNA at internucleosomal regions (nucleosomes contain 180bp of DNA in addition to histone proteins) and may leak out of apoptotic bodies. These oligonucleosomal fragments can be visualised as a characteristic DNA ladder on agarose gels (Wyllie, 1980). It is likely that degradation of proteins involved in maintaining chromatin structure (such as histone H2A by the ubiquitin degradation pathway (Marushige and Marushige, 1996)) results in increased access of the internucleosomal DNA to nuclease attack(Delic *et al,* 1993).

The identity of the endonuclease responsible for cleavage of chromatin into oligonucleosomes has proven to be elusive although several groups have suggested candidates that have included DNAseI (Peitsch *et al,* 1993), DNAseII (Barry and Eastman, 1993) and DNAses designated alpha, beta and gamma (Shiokawa *et al,* 1994). It is a common finding that the endonuclease activity has a requirement for Ca^{2+}/Mg^{2+} and is inhibitable with Zn^{2+} ions (Zhang *et al,* 1995; Sokolova *et al,* 1995; Ribeiro and Carson, 1993; Arends *et al,* 1990) and although endonucleases with these properties have been purified from several cell types, no agreement has been reached relating to the optimal pH of an apoptosis specific endonuclease which acts specifically at internucleosomal DNA. A more detailed discussion of this subject appears in (Eastman, 1995).

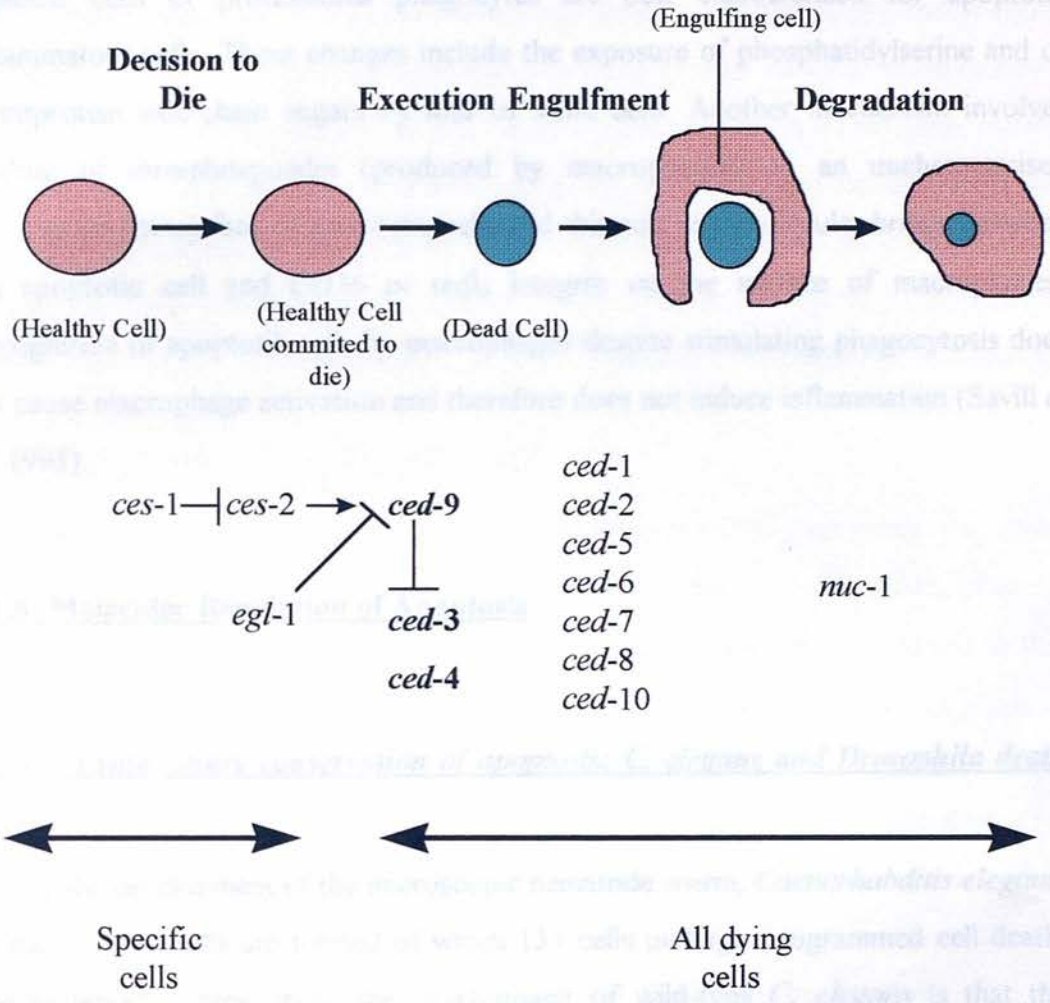


Fig. 1.2: *C. elegans* Programmed Cell Death Genes. (Adapted from Steller, 1995).

1.1.4.3 Surface Changes

Cell surface changes during apoptosis that promote recognition and phagocytosis by adjacent cells or professional phagocytes are best characterised for apoptotic inflammatory cells. These changes include the exposure of phosphatidylserine and of glycoprotein side-chain sugars by loss of sialic acid. Another mechanism involves binding of thrombospondin (produced by macrophages) to an uncharacterised receptor on the surface of apoptotic cells and this acts as a molecular bridge between the apoptotic cell and CD36 or $\alpha_3\beta_3$ integrin on the surface of macrophages. Recognition of apoptotic cells by macrophages despite stimulating phagocytosis does not cause macrophage activation and therefore does not induce inflammation (Savill *et al*, 1993).

1.1.5 Molecular Regulation of Apoptosis

1.1.5.1 Evolutionary conservation of apoptosis: C. elegans and Drosophila death genes.

During the development of the microscopic nematode worm, *Caenorhabditis elegans*, a total of 1090 cells are formed of which 131 cells undergo programmed cell death. The remarkable thing about the development of wild-type *C. elegans* is that the parent-daughter cell relationship of each cell is identical from individual to individual. Using Nomarski optics it is possible to identify each cell of the transparent worm and the precise fate, in terms of differentiation state or cell death, can be predicted with absolute precision. By having access to this knowledge it was possible to identify several mutant worm strains which were cell death defective (*ced*) (Hedgecock *et al*, 1983). Combinatorial analyses of these mutations revealed a cell death pathway where some genes control the commitment of individual cells to die (e.g. *ces*-1, *ces*-2, *egr*-1), some carry out the death program, whilst others are responsible for the engulfment and disposal of cell corpses (Fig. 1.2). Two genes (*ced*-3 and *ced*-4) are absolutely

required for the execution of all cell death as mutant animals contain excess cells (Ellis and Horvitz, 1986). The *ced*-9 loss of function mutants were embryonic lethal as many cells died, indicating that *ced*-9 prevents ectopic cell death. A rare *ced*-9 gain of function mutant (n1950) showed defects in the deletion of cells (Hengartner *et al,* 1992). Other genes identified did not affect the decision of cells to die, rather there were defects in the processing of cell corpses in these animals. Most of these mutants showed defects in the engulfment of cell corpses (Ellis *et al,* 1991). One gene, *nuc*-1, encoding an endonuclease, was not required for the execution of cell death or engulfment of corpses, but corpses were not degraded completely once engulfed by surrounding cells (Hevelone and Hartman, 1988; Sulston, 1976). It is not known whether this enzyme is similar to the Ca^{2+}/Mg^{2+}-dependent endonuclease activated in mammalian apoptosis.

Strikingly, following the cloning of cDNAs for several of the *ced* genes, sequence comparison studies found similarity between *ced*-3 and a mammalian protein, interleukin-1β-convertase (ICE), a protease which was known to be involved in the processing of pro-IL-1β to its mature secreted form. This protease had an unusual specificity for aspartate residues (single-amino acid code: D) and its overexpression in Rat1 fibroblasts resulted in apoptosis (Yuan *et al,* 1993). Further, *ced*-9 was shown to be structurally homologous to the mammalian protein Bcl-2 (Hengartner and Horvitz, 1994). Despite the fact that no mammalian homologue of *ced*-4 has yet been found, the obvious functional similarity between *ced*-9 and Bcl-2 and the discovery of more cysteine proteases implicated in apoptosis (see below) indicated that the cell death machinery has been conserved in evolution from nematode to human.

This evolutionary conservation has been underlined by the discovery *in Drosophila melanogaster* of a small peptide, REAPER, that is expressed early (within 1-2 hours of a death stimulus) in cells destined to die. Deletion mutations of the *reaper* gene abolish all programmed cell deaths during development. In addition, cell deaths caused by mutations in genes involved in morphogenesis as well as those following moderate-dose irradiation are blocked in the absence of REAPER (White *et al,* 1994).

REAPER expression causes apoptosis by activation of an ICE-like protease and is also responsible for ceramide generation (Pronk *et al*, 1996). A REAPER-like domain has been found in the cytoplasmic domains of the apoptosis-inducing cell surface receptors, APO1/Fas/CD95 and TNFR-1 (Golstein *et al*, 1995; Itoh and Nagata, 1993). Mutations in this 'death domain' are sufficient to prevent activation of cell death pathways caused by binding of ligand to these receptors (Tartaglia *et al*, 1993; Itoh and Nagata, 1993). Despite the increased complexity and functional redundancy of mammalian cell death components, together with the slow pace of genetic manipulation in higher organisms, the genetic information derived from *C. elegans* and *Drosophila* will prove to be invaluable in the study of mammalian apoptosis.

1.1.5.2 Organisation of the Apoptotic Program.

The final events in the apoptotic process are believed to be determined by the activity of a set of gene products that regulate effector mechanisms. Genes encoding products involved in effector mechanisms constitute a stereotyped "program" of events which can be triggered in various ways depending on the cell type and state of differentiation (Fig. 1.3). The existence of such a final common pathway would be predicted to explain the similarity in morphology of many types of cells undergoing apoptosis. There is now strong evidence that some of the genes known to be able to cause apoptosis (the *ced*-3-related cysteine proteases, see below) are directly involved in cell dismantlement and are conserved between the nematode and humans. To what extent these molecular events are organised in linear cascades or parallel independent processes, that together result in the phenomenon we know as apoptosis, is not yet clear. However, there is known to be a degree of functional redundancy between the components. An obligate gene, one whose absence abolishes apoptosis in all situations, has not been found.

Genes acting upstream of this effector pathway are critical in determining whether this program of events is switched on or off in a particular cell. These genes may also be suitable candidates for manipulation in future therapeutic strategies designed to enhance or diminish apoptotic cell death in clinical situations such as cancer or tissue

enhance or diminish apoptotic cell death in clinical situations such as cancer or tissue transplantation respectively. Some of these genes (e.g. c-myc, bcl-2, p53) are already known as oncogenes and oncosuppressor genes and appear to determine susceptibility of particular cells to cell death.

Three main strategies by which the effector molecules of apoptosis can be activated in various cells can be identified on the basis of treatment of cells with mRNA or protein synthesis inhibitors such actinomycin D (actD) or cycloheximide (CHX)(Cohen *et al,* 1992; Cohen, 1991):

Induction mechanisms are blocked by actD or CHX. Apoptosis in these systems (e.g. glucocorticoid-treated (Wyllie *et al,* 1984), irradiated or antigen stimulated thymocytes) is therefore dependent on the synthesis of new gene products, the likely functions of which may be to activate effector mechanisms. In γ-irradiated thymocytes an apoptotic signal persists within the cells for a least 6 hours and may represent irreversible activation of effector pathways that are still dependent on new protein synthesis at points further down the pathway (Sellins and Cohen, 1987);

Transduction of the apoptotic stimulus to the effector pathway, for example in cytotoxic T lymphocyte or NK cell-mediated cytotoxicity, is not inhibitable by actD or CHX (Duke *et al,* 1983). In nearly all cell types the essential molecular components of apoptotic cell death therefore appear to be constitutively present in most nucleated cell types, including some tumour cells.

Release of cells into apoptosis can be initiated by treatment with actD or CHX (Rotello *et al,* 1991; Cohen, 1991) in some cell types (Cohen, 1991) and certain tumour cells (Cohen, 1991; Martin et al, 1990) implying that apoptosis may be repressed by a gene product(s) with presumably a short half-life.

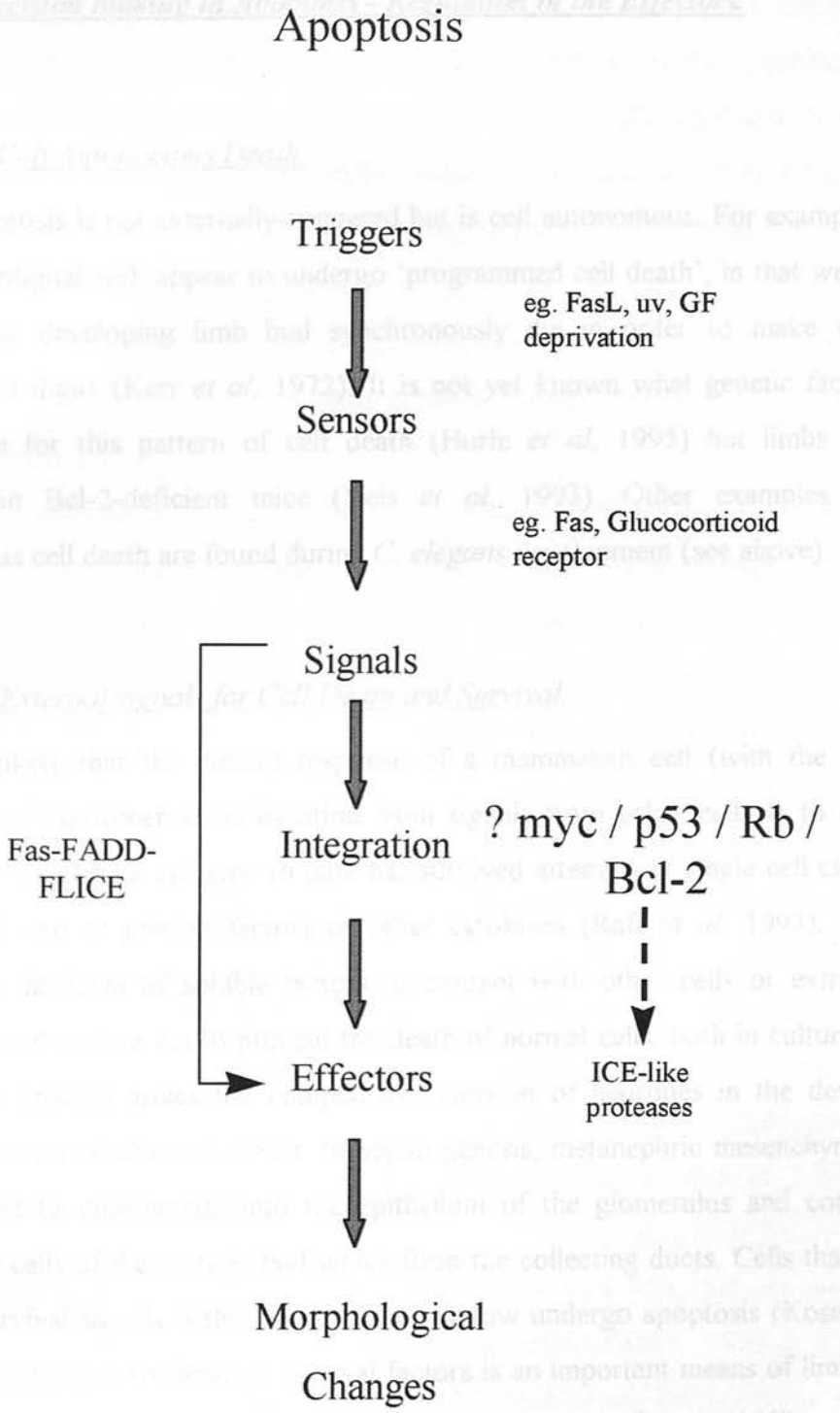


Fig. 1.3: Organisation of the Apoptotic Program.

1.1.5.3 Decision making in Apoptosis - Regulation of the Effectors.

1.1.5.3.1 Cell Autonomous Death.

Some apoptosis is not externally-triggered but is cell autonomous. For example, cells of the interdigital web appear to undergo 'programmed cell death', in that web space cells in the developing limb bud synchronously die in order to make way for independent digits (Kerr *et al*, 1972). It is not yet known what genetic factors are responsible for this pattern of cell death (Hurle *et al*, 1995) but limbs develop normally in Bcl-2-deficient mice (Veis *et al*, 1993). Other examples of cell autonomous cell death are found during *C. elegans* development (see above).

1.1.5.3.2 External signals for Cell Death and Survival.

It seems likely that the default response of a mammalian cell (with the possible exception of blastomeres) to isolation from signals from other cells is to undergo apoptosis. No normal cell type to date has survived attempts at single cell cloning in medium devoid of growth factors or other cytokines (Raff *et al*, 1993). Survival signals, in the form of soluble factors or contact with other cells or extracellular matrix must therefore act to prevent the death of normal cells, both in culture and in vivo. This process drives the competitive selection of neurones in the developing nervous system (Raff *et al*, 1993). In nephrogenesis, metanephric mesenchymal cells are induced to differentiate into the epithelium of the glomerulus and convoluted tubules by cells of the ureteric bud which form the collecting ducts. Cells that do not receive survival signals within certain time window undergo apoptosis (Koseki *et al*, 1992). Competition for limiting survival factors is an important means of limiting the size of cell populations in constantly renewing tissue such as epithilia and bone marrow. Only a proportion of the daughters of stem cells survive to become terminally differentiated. Survival signals, including soluble cytokines and paracrine factors are required and determine cell number. Terminally differentiated cells also require survival support and these signals often differ from those required by immature cells. The epithelia of hormone-dependent tissues, such as prostate, breast,

endometrium and adrenal gland all undergo involution by apoptosis if the source of hormone is withdrawn (Arends and Wyllie, 1991; Kerr *et al*, 1972). Likewise, cells of the immune system are sensitive to the withdrawal of specific cytokines. In addition, differentiated epithelial and endothelial cells, in contrast to fibroblasts, require attachment to a basement membrane via integrin-matrix interactions for survival as disruption of integrin binding results in apoptosis. (Frisch and Francis, 1994; Meredith, J.E. *et al*, 1993). Escape from these site-specific attachments is therefore of prime importance in epithelial neoplastic progression and metastasis.

For tissue homeostasis to occur, a balance between cell survival and cell death must be struck and this therefore implies that communication between cells must also be capable of actively causing apoptosis. Ligand binding to cell surface receptors can mediate cell survival or cell death. In some cases interaction between cells may involve simultaneous signals promoting death, survival or growth. In the dual signal model of antigen-mediated activation of lymphocytes, antigen recognition must also be accompanied by co-stimulatory signals. For example, antigen bound to surface immunoglobulin tends to cause apoptosis of B cells unless a second signal is provided, for example, through CD40. The ligand for CD40 is present on activated, but not resting helper T cells. Therefore B cell clones with immunoglobulin specificities for self antigen are not rescued and are deleted (Tsubata *et al*, 1993). Similarly, the activation of T cells requires co-stimulation from antigen presenting cells in addition to the activation of the T cell receptor (TCR)/CD3 complex (Smith *et al*, 1989).

The TNF receptor family (Fig. 1.4) includes several members that are involved in activation or inhibition of cell death including Fas/APO-1/CD95, TNFR-1, CD40 and low affinity-NGFR (Nagata and Golstein, 1995; Itoh *et al*, 1991). While the main activity of Fas is to cause death, TNFα can also cause fibroblast proliferation and prostaglandin synthesis amongst other functions (Tartaglia and Goeddel, 1992). All the TNFR family of molecules contain between three to six cysteine-rich, conserved extracellular domains. However, TNFR-1 and Fas possess similar stretches of about 70 amino acids in their cytoplasmic portions and these 'death domains' are necessary

and sufficient for transduction of the death signal upon binding of TNF-α or Fas-ligand (FasL) respectively (Tartaglia *et al*, 1993; Itoh and Nagata, 1993). The Fas antigen may also be stimulated by cross-linking with activating antibody or by soluble FasL released from activated cytotoxic cells (Tanaka *et al*, 1996; Ogasawara *et al*, 1993; Yonehara *et al*, 1989). Fas is expressed weakly in most tissues but at much higher levels in liver, heart, lung, kidney, ovary (WatanabeFukunaga *et al*, 1992b) and most mouse, but not human, thymocytes other than immature $CD4^-/CD8^-$ cells (Ogasawara *et al*, 1995; Andjelic *et al*, 1994). Additionally, Fas is upregulated on activated peripheral lymphocytes (Trauth *et al*, 1989). The spontaneous murine mutations of Fas (*lpr* (lymphoproliferation)), and FasL (*gld* (generalised lymphoproliferative disease)) cause accumulation of activated lymphocytes. This results in lymphadenopathy and splenomegaly, and on the MRL strain background, autoimmune conditions such as glomerulonephritis and arthritis (Izui *et al*, 1984; Cohen and Eisenberg, 1991). Since positive and negative selection in the thymus is not disturbed in *lpr* or *gld* mice, these immune system defects indicate that the Fas-FasL system is involved in peripheral clonal deletion and deletion of activated lymphocytes (Nagata and Golstein, 1995). Further, FasL is upregulated on the surface of activated cytotoxic T lymphocytes ($CD8^+$ or $CD4^+$ T_H1 cells) (Nagata and Golstein, 1995; Suda *et al*, 1993), which are the main effectors against virally infected cells. The importance of the Fas system as a mechanism of cytotoxicity was underlined in elegant experiments that showed that $CD8^+$ CTL killing by Fas and perforin was additive and sufficient to account for all cell-mediated cytotoxicity, at least in the short term *in vitro* (Lowin *et al*, 1996; Kagi *et al*, 1994). The importance of Granzyme B, an aspartate-specific serine protease found in cytotoxic cell granules and that acts synergistically with perforin, is not clear (Trapani, 1995).

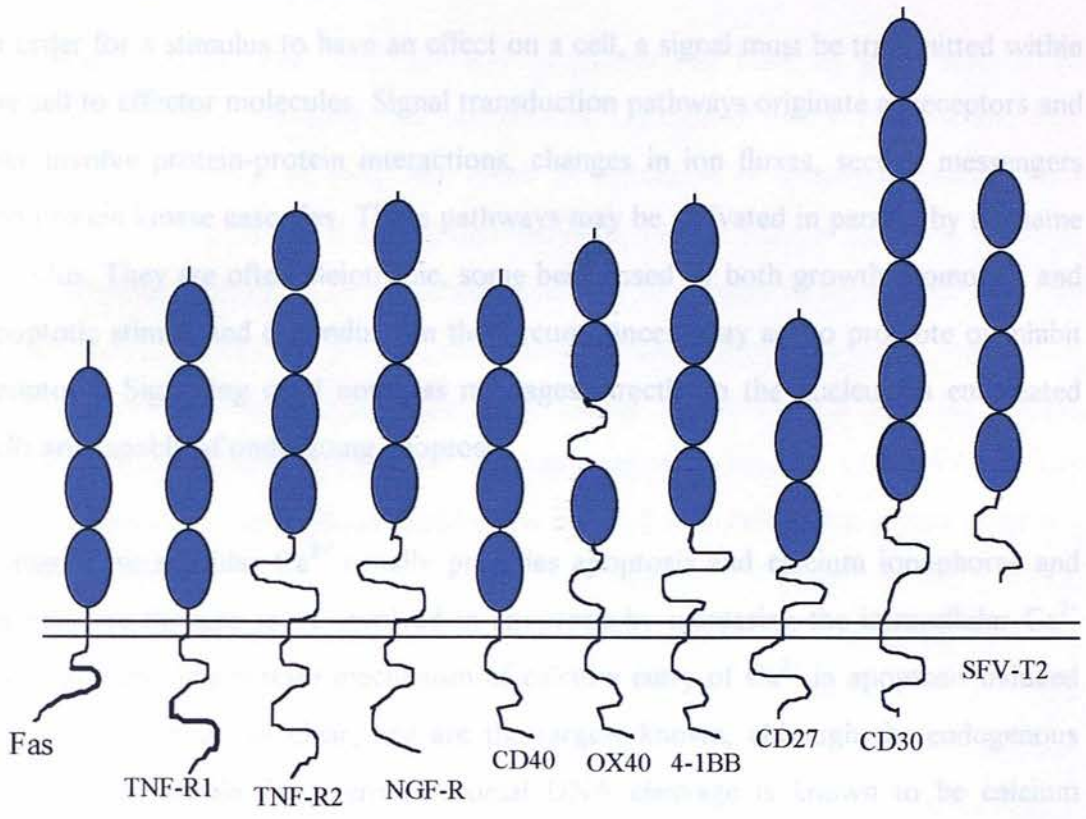


Fig. 1.4: Comparison of the Structures of the TNF-R Superfamily. (From Nagata and Golstein, 1995). The extracellular domains are strongly similar between family members. The two death promoting members, Fas and TNFR-1, have similar, *Reaper*-like death intracellular domains that transmit death initiating signals (via TRADD or FADD and FLICE) to molecules in the cytoplasm.(bold).

1.1.5.3.3 Signalling pathways.

In order for a stimulus to have an effect on a cell, a signal must be transmitted within the cell to effector molecules. Signal transduction pathways originate at receptors and may involve protein-protein interactions, changes in ion fluxes, second messengers and protein kinase cascades. These pathways may be activated in parallel by the same stimulus. They are often pleiotropic, some being used by both growth promoting and apoptotic stimuli and depending on the circumstances, may act to promote or inhibit apoptosis. Signalling need not pass messages directly to the nucleus as enucleated cells are capable of undergoing apoptosis.

A rise in intracellular Ca^{2+} usually precedes apoptosis and calcium ionophores and perforin are thought to be involved in apoptosis by increasing the intracellular Ca^{2+} concentration. The precise mechanism of calcium entry of Ca^{2+} in apoptosis induced by other stimuli is not clear, nor are the targets known, although the endogenous nuclease responsible for internucleosomal DNA cleavage is known to be calcium dependent. It is possible that the response to calcium is mediated by calcium-calmodulin-dependent protein kinase or calpain although these enzymes have many substrates. The action of Ca^{2+} is not specific to apoptosis however as changes in cytosolic calcium are involved in other cell processes such as muscle contraction and T cell activation. Calcium is also required for the activation of protein kinase C (PKC) by another second messenger, diacyl glycerol, a product of phospholipase C-mediated hydrolysis of the membrane lipid phosphoinositol 4,5-bisphosphate (PIP_2). Phospholipase C is regulated by G proteins whose activity is often controlled by cell-surface receptors such as the TCR/CD3 complex. Artificial stimulation of PKC by a combination of ionomycin (a calcium ionophore) and a phorbol ester such as phorbol myristate acetate (PMA) is known to cause apoptosis in some systems, but is protective in others. A signalling pathway involving arachidonic acid metabolism has been implicated in thymocyte apoptosis and the *ras-raf* protein kinase cascade, a reasonably well characterised means of communication of signals from cell surface

receptors to transcription factors in the nucleus, has been found to inhibit apoptosis when activated in fibroblasts (Troppmair *et al*, 1992; Arends and Harrison, 1994; Arends *et al*, 1993).

Signalling from the Fas and TNFR-1 molecules has been of great interest to researchers looking into apoptosis mechanisms. Ligation of either TNFR-1 and Fas, and indeed irradiation of cells, can result in activation of sphyngomyelinases and production of ceramide (HaimovitzFriedman *et al*, 1994; Cifone *et al*, 1994; Obeid *et al*, 1993). This pathway is activated independently of damage in the nucleus as it can be activated in purified membrane preparations. Treatment of cells with C2-ceramide, a cell-permeable synthetic analogue, can induce apoptosis and this can bypass pharmacological blockade of acidic sphyngomyelinase (Higuchi *et al*, 1996). The means of action of ceramide in apoptosis is not well understood but it may involve a ceramide-activated serine/threonine protein kinase (Cifone *et al*, 1995; HaimovitzFriedman *et al*, 1994) or a ceramide-initiated *ras* signalling pathway (Gulbins *et al*, 1995). The ceramide pathway may interact with other signalling pathways as several groups have observed that ceramide production and apoptosis following Fas or TNFR-1 ligation can be inhibited by the PKC agonist PMA (Tepper *et al*, 1995; HaimovitzFriedman *et al*, 1994; Obeid *et al*, 1993).

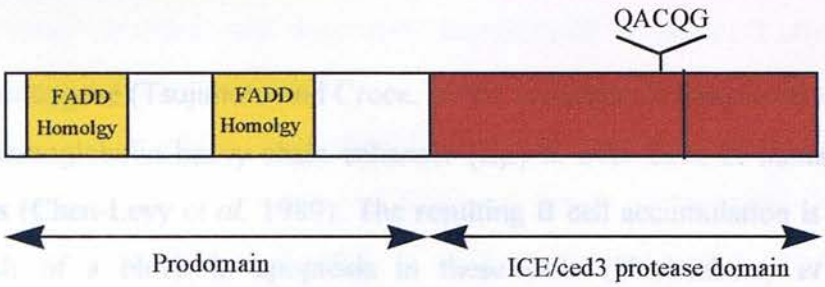
Despite the detail that has been collected concerning the workings of signal transduction pathways, many questions remain to answered with regard to their role in apoptosis. In particular, it remains to be seen how these pathways connect up with and activate effector molecules. However, in the case of Fas and TNFR-1, exciting recent research has demonstrated a direct physical link between the cytoplasmic death domains and a death-promoting signal complex consisting of an adapter molecules containing death domains, MORT1/FADD (Fas associated protein with death domain) for Fas (Chinnaiyan *et al*, 1995; Boldin *et al*, 1995) and TRADD for p55 TNFR-1 (Hsu *et al*, 1995), and another protein, MACH/FLICE, which binds to these complexes and contains a domain with significant homology to the *ced*-3/ICE-like proteases (Fig. 1.5). It is likely that ligation of Fas or TNFR-1 induces the cysteine

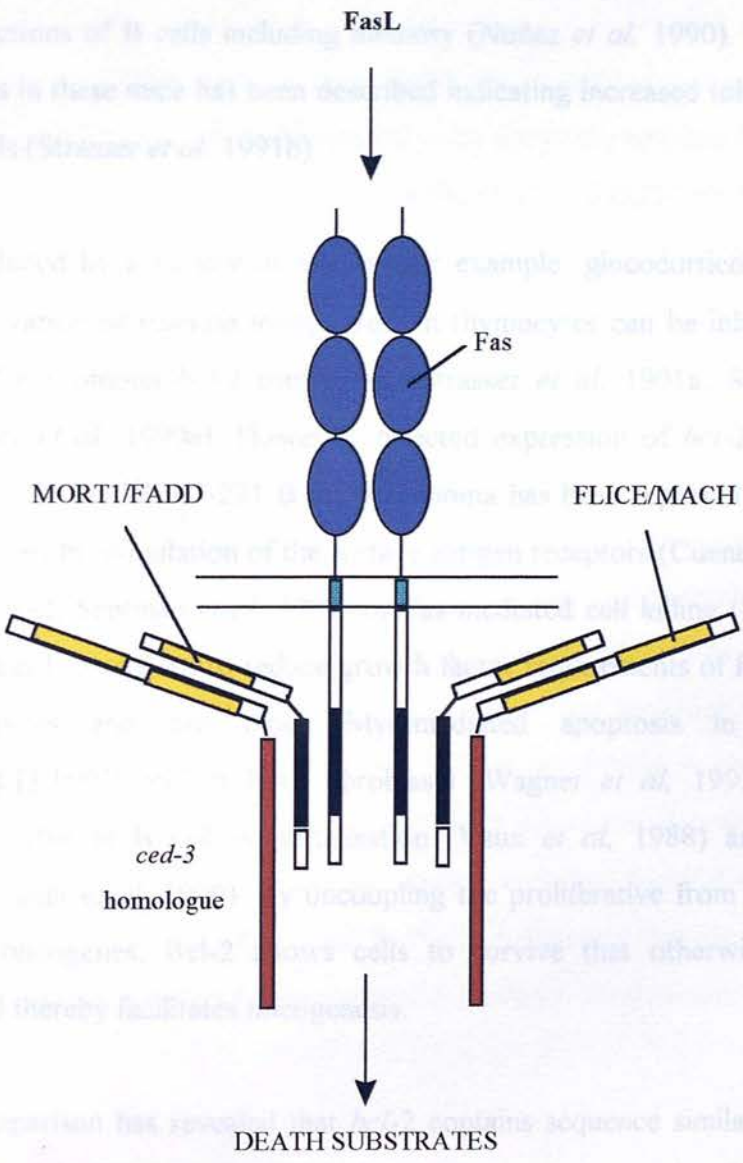
cysteine protease activity of MACH/FLICE by causing autocatalysis and release of the active cysteine protease domain (Muzio *et al,* 1996; Boldin *et al,* 1996). Such proteases are thought to be the direct effectors of apoptosis.

1.1.5.3.4 Signal Integration.

As discussed above, both death and survival signals activate signal transduction pathways that regulate apoptosis. How these pathways are connected to the effector mechanisms in order to activate or prevent cell death is still unclear in most cases. However some of the molecules that are involved in making the decision of a whether a cell should survive or die have been identified. Given that cancer is essentially a problem of cell population control and that one means of controlling cell number is by deleting excess cells, it is hardly surprising that many of the genes that regulate the susceptibility of cells to undergo apoptosis were first identified as oncogenes (e.g. *bcl*-2, c-*myc*) or oncosuppressor genes (e.g. *Rb*, p53).

Fig. 1.5 (overleaf)**: Signal Transduction by Fas via FLICE.** A) Structure of FLICE (Muzio *et al*, 1996). The QACQG sequence in the Ced-3 homology domain is similar to that in the active site (QACRG) of the other proteases. B) Activation of FLICE (Boldin *et al*, 1996). FasL binds to the extracellular portion of Fas and this causes the intracellular domain to bind to FLICE via FADD(MORT1). This interaction causes the activation of FLICE probably by autocatalytic cleavage of the ICE-like protease domain from the prodomain.


QACQG
FADD Homolgy
FADD Homolgy
Prodomain
ICE/ced3 protease domain


FasL
Fas
MORT1/FADD
FLICE/MACH
ced-3
homologue
DEATH SUBSTRATES

1.1.5.4 A possible mechanism for signal integration: The bcl-2 gene family. (Fig. 1.6)

The *bcl*-2 oncogene (Tsujimoto and Croce, 1986) undergoes a translocation, t[14;18], to the immunoglobulin heavy chain enhancer (Eμ) in over 80% of human follicular lymphomas (Chen-Levy *et al*, 1989). The resulting B cell accumulation is thought to be a result of a block in apoptosis in these cells (Hockenberry *et al*, 1991; Hockenberry *et al*, 1990; Nuñez *et al*, 1990; Tsujimoto *et al*, 1987). Eμ-*bcl*-2 transgenic mice with high levels of Bcl-2 expression in cells of the B lineage also show a similar pattern of follicular lymphomas (McDonnell and Korsmeyer, 1991) as well as prolongation of cell survival (McDonnell *et al*, 1990; McDonnell *et al*, 1989) and most functions of B cells including memory (Nuñez *et al*, 1990). Production of autoantibodies in these mice has been described indicating increased tolerance of self-reactive B cells (Strasser *et al*, 1991b).

Apoptosis induced by a variety of agents (for example: glucocorticoids, radiation, cytokine deprivation or calcium ionophores) in thymocytes can be inhibited by Eμ-*bcl*-2 or by *lck* promoter-*bcl*-2 transgenes (Strasser *et al*, 1991a; Sentman *et al*, 1991a; Strasser *et al*, 1990a). However, directed expression of *bcl*-2 to immature thymocytes and in the WEHI-231 B cell lymphoma has been reported not to inhibit apoptosis induced by stimulation of the surface antigen receptors (Cuende *et al*, 1993; Fanidi *et al*, 1992; Sentman *et al*, 1991) or Fas-mediated cell killing (Strasser *et al*, 1995). Bcl-2 has been shown to reduce growth factor requirements of fibroblasts and other cell types and can block Myc-mediated apoptosis in CHO cells (BISSONNETTE1992) and in Rat1 fibroblasts (Wagner *et al*, 1993). Bcl-2 co-operates with Myc in B cell immortalisation (Vaux *et al*, 1988) and in tumour formation (Strasser *et al*, 1990). By uncoupling the proliferative from the apoptotic functions of oncogenes, Bcl-2 allows cells to survive that otherwise would be eliminated and thereby facilitates oncogenesis.

Sequence comparison has revealed that *bcl*-2 contains sequence similarity to the *C. elegans* cell death inhibitory gene, *ced*-9. A *bcl*-2 transgene rescued cells from ectopic

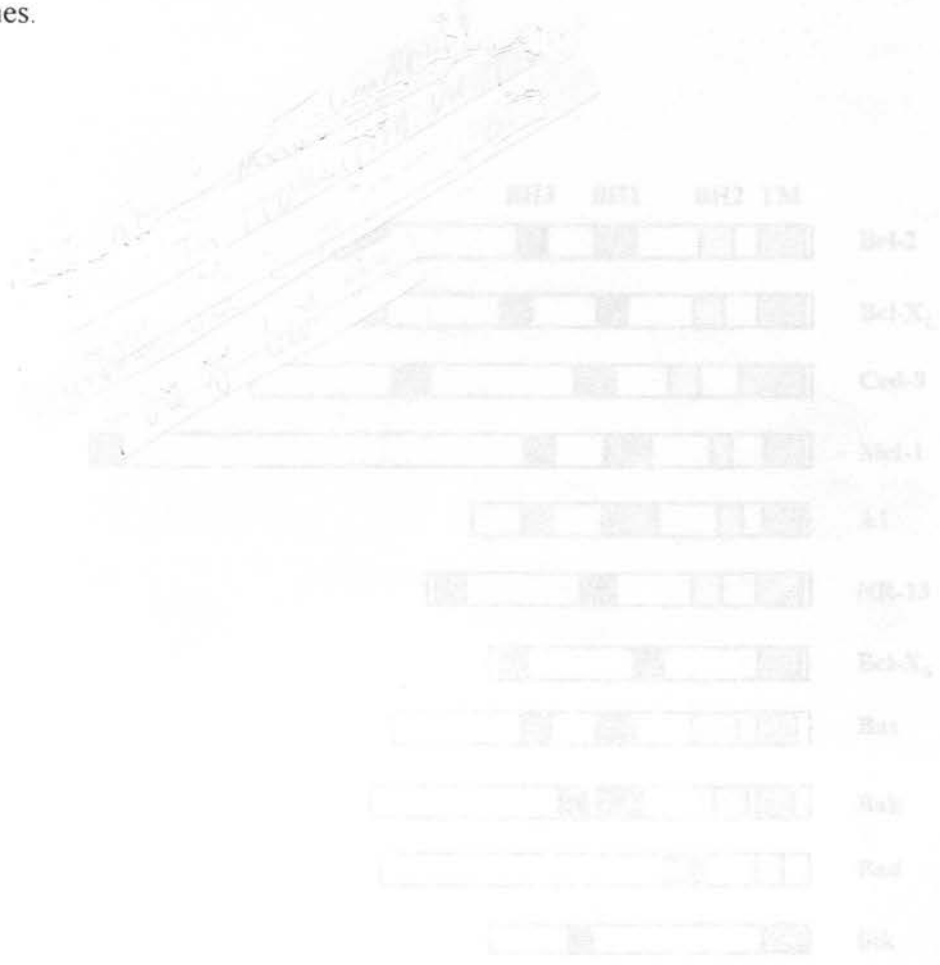
cell death in ced-9 loss-of-function mutants and the conclusion was drawn that these two genes were structural and functional homologues (Hengartner and Horvitz, 1994). Bcl-2 expression is not, however, critical for survival in all mammalian tissues. Mice with targeted knockout alleles (*bcl*-2 -/-) in the main develop normally, adult mice dying with polycystic kidneys and fulminant lymphoid apoptosis (Veis *et al*, 1993). However knockout of a related gene, *bcl*X, results in the death of homozygous null embryos at stage E13 accompanied by massive cell death of neurones and immature haematopoietic cells (Motoyama *et al*, 1995). The long splice variant, $BclX_L$ which is protective against cell death, is expressed at high levels in brain (Boise *et al*, 1993).

Two protein domains (BH1 and BH2) in Bcl-2 are conserved in both Ced-9 and $BclX_L$ and are known to be involved in inhibition of apoptosis and heterodimerisation with the proapoptotic Bcl-2 homologue, Bax (Yin *et al*, 1994a; Oltvai *et al*, 1993a). Using the BH1 and BH2 sequences of the Bcl-2-related proteins it was possible to clone a further gene, Bak, that enhances the response to apoptotic stimuli (Kiefer *et al*, 1995; Chittenden *et al*, 1995). A dynamic equilibrium exists between Bcl-2/Bax (and $BclX_L$/Bax) heterodimers and homodimerisation of Bcl-2 (or $BclX_L$) and Bax. Bax homodimers are proapoptotic (Yin *et al*, 1994a), whereas suppression of apoptosis by Bcl-2 requires heterodimerisation with a killer protein such as Bax (Yin *et al*, 1994b). It is thought that the relative levels of killer and protective molecules determines the susceptibility of cells to an apoptotic stimulus (Fig. 1.7). This is supported by the discovery of proteins that have no obvious sequence homology to, and that interact with, Bcl-2 family members in yeast two-hybrid protein interaction screens. Increases in the concentration of Bag/Bcl-2 heterodimers tends to increase the resistance of JURKAT cells to apoptotic stimuli, including anti-Fas (Takayama *et al*, 1995), whereas Bad binds to Bcl-2 and $BclX_L$ and displaces Bax thus enabling the formation of a greater number of proapoptotic Bax/Bax homodimers (Yang *et al*, 1995).

The anti-apoptotic family members, Ced-9, Bcl-2, $BclX_L$, Mcl-1, A1 and Nr-13, all contain an additional regional of homology (BH4) near the N-terminus that is essential for anti-apoptotic activity but is not required for binding to Bax (Hanada *et al*, 1995; Borner *et al*, 1994). However, Bax does not use its BH1 or BH2 domains to form homo- or heterodimers but uses a separate BH3 domain for dimerisation with itself and Bcl-2 (Boyd *et al*, 1995; Zha *et al*, 1996). Although Bcl-2 contains a similar BH3 domain, deletion of the Bcl2 BH3 domain does not affect protein function but replacement of a small region containing the Bcl-2 BH3 domain with a 22 amino acid peptide containing the BH3 domain from Bax is sufficient to transfer death-promoting properties to Bcl-2 (Hunter and Parslow, 1996). Similarly, overexpression of a minimal deletion mutant Bak protein, containing little more than the BH3 domain, was sufficient to promote apoptosis (Chittenden *et al*, 1995a). Since NBK/Nip4/Bik does not possess BH1 or BH2, nor any significant homology to any other region of Bcl-2 family members except at the 9 amino acid BH3 motif (Boyd *et al*, 1995), the BH3 of proapoptotic family members Bik, Bak, Bax and $BclX_S$ (an alternatively spliced form of BclX that does not contain BH1 or BH2) represents a critical 'suicide domain'.

It can be envisaged from the above observations that both apoptotic and survival signals perturb the balance between the proapoptotic and antiapoptotic dimers and that this integration of signals influences a cell's response to apoptotic stimuli (Vaux *et al*, 1994). However, it is still undecided which of the two groups of factors, the proapoptotic ($BclX_S$, Bak, Bax, Bik) and the antiapoptotic (Bcl-2, $BclX_L$), are responsible for passing on their decisions to effector molecules or how this is achieved. One model suggests that Bax and Bak are killers in their own right with Bcl-2 and $BclX_L$ acting to inhibit their activity. Another model suggests that Bax and Bak have no function other than to bind Bcl-2 or $BclX_L$ and therefore block their protective activity. An attractive alternative, given that the protectors and the killers have different functional domains, is that they have interrelated but distinct functions and that relative protein concentrations determine the outcome for the cell (Farrow and Brown, 1996). Confirmation of one of these models awaits elucidation of

immediate downstream effectors and the mechanisms by which they are activated and careful analysis of phenotypes of mice containing multiple knockouts of Bcl-2 family genes.



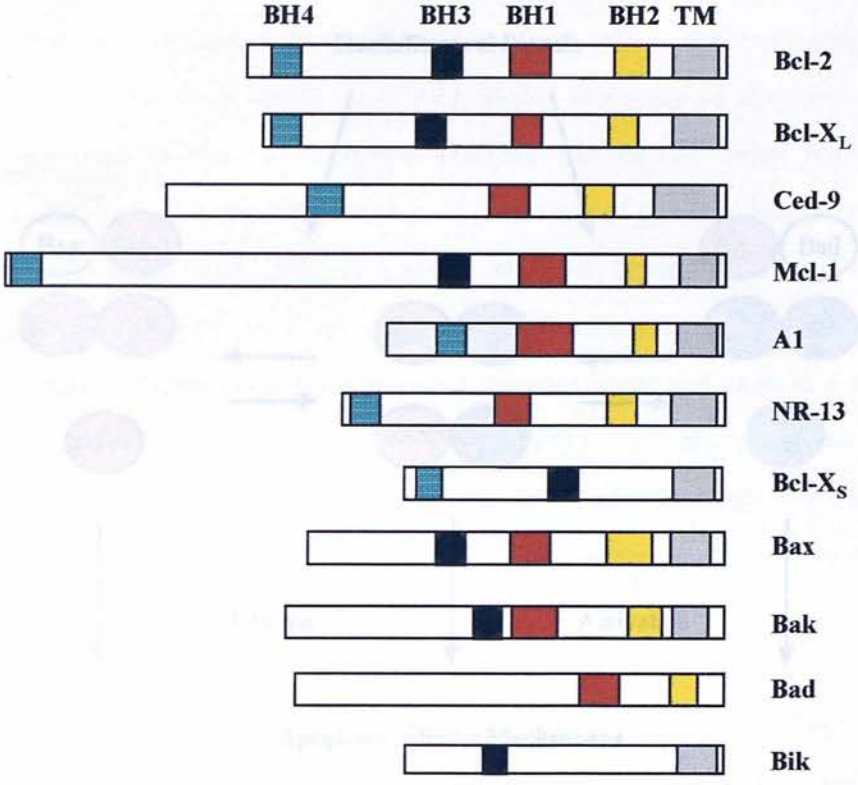


Fig. 1.6: The Bcl-2/Ced-9 Superfamily. Domain structure of the Bcl-2 family. BH: Bcl-2 homology domain. TM: transmembrane domain. (From Zha *et al*, 1996).

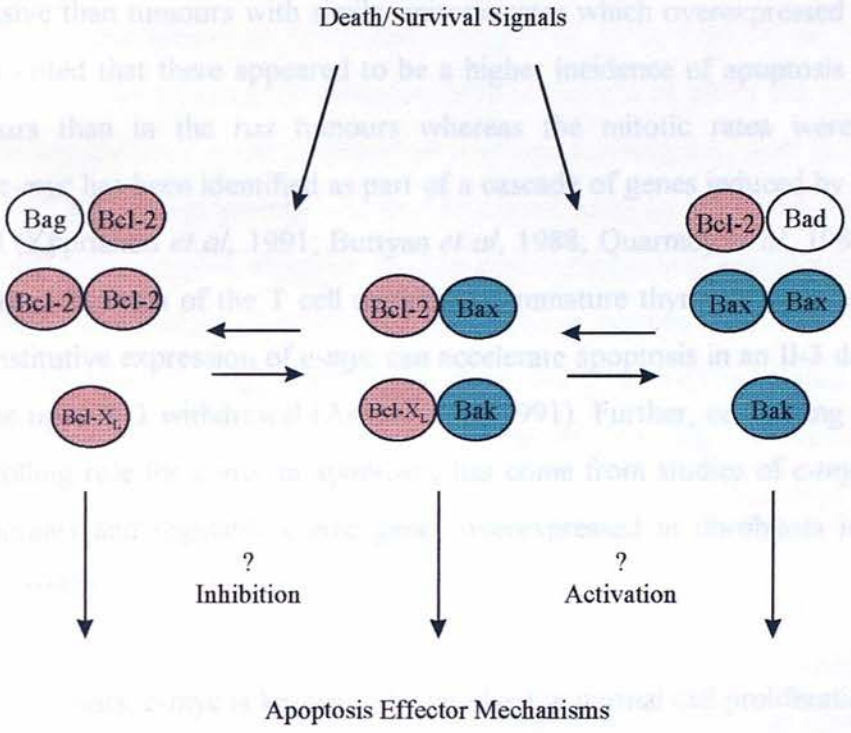


Fig. 1.7: Model for Integration of Death and Survival Signals by the Bcl-2 Family. (Adapted from Farrow and Brown, 1996). The balance between dimers containing pro-apoptotic and inhibitory family members is seen in this model as being regulated by environmental cues representing death and survival factors. The tipping of the balance to the left causes an increase in resistance to death signals and *vice versa*. This therefore determines the susceptibility of cells to death signals. It is not known how the Bcl-2 family members interact with the effector molecules (ICE-like proteases) to regulate their activity, but both activation and inhibition may occur.

1.1.5.5 The Role of c-myc in Apoptosis.

A number of lines of evidence have pointed to the central role of the oncogene c-*myc* in many forms of apoptosis. Firstly, Wyllie et al (1987) noticed that experimental rodent fibrosarcomas overexpressing c-*myc* appeared to grow more slowly and were less aggressive than tumours with similar mitotic rates which overexpressed T24 Ha-*ras*. It was noted that there appeared to be a higher incidence of apoptosis in the c-*myc* tumours than in the *ras* tumours whereas the mitotic rates were similar. Secondly, c-*myc* has been identified as part of a cascade of genes induced by hormone withdrawal (Kyprianou *et al*, 1991; Buttyan *et al*, 1988; Quarmby *et al*, 1987) and is induced after activation of the T cell receptor of immature thymocytes (Riegel *et al*, 1990). Constitutive expression of c-*myc* can accelerate apoptosis in an Il-3 dependent myeloid line upon Il-3 withdrawal (Askew *et al*, 1991). Further, convincing evidence for a controlling role for c-*myc* in apoptosis, has come from studies of c-*myc*, c-*myc* deletion mutants and regulable c-*myc* genes overexpressed in fibroblasts in culture (Evan *et al*, 1992).

In normal fibroblasts, c-myc is known to be involved in normal cell proliferation and is one of the immediate early growth response genes. While Myc protein levels peak at three hours after mitogen stimulation (3-6 x 10^3 molecules per cell), unlike other genes of this group, levels persist in cells (1-3 x 10^3 molecules per cell) so long as they remain in a proliferative state. The half-lives of c-*myc* mRNA and protein are very short and endogenous Myc levels fall rapidly when mitogenic stimuli are withdrawn, independent of cell cycle stage (Waters *et al*, 1991; Moore and Evan, 1987; Dean *et al*, 1986). Quiescence (G0) is associated with undetectable levels of Myc. Enforced Myc expression can stimulate quiescent G0 fibroblasts into the cell cycle (Eilers *et al*, 1989) although c-*myc* antisense oligonucleotides can inhibit progression into S phase and not entry into G1 from G0 (Heikkila *et al*, 1987). Myc is a basic-helix-loop-helix-leucine zipper (b-HLH-LZ) protein and forms a heterodimeric complex with Max to form a transcription factor (Amati *et al*, 1993; Murre *et al*, 1989; Landshultz *et al*, 1988; Blackwood and Eisenman, 1991) which binds to and

can activate transcription from a consensus DNA binding site called the E-box: CAC(G/A)TG (Prendergast and Ziff, 1991; Blackwood and Eisenman, 1991; Blackwell *et al*, 1990). Max can be displaced from Myc-Max complexes by binding to either of the related proteins Mad or MxiI and these interactions negatively affect transcription mediated by Myc (Zervos *et al*, 1993; Ayer *et al*, 1993). Only a few genes have been shown to be transcriptionally regulated by Myc *in vivo* (Reisman *et al*, 1993; Bello-Fernandez *et al*, 1993; Benvenisty *et al*, 1992; Eilers *et al*, 1991) and although there is little mechanistic evidence, Myc is thought to affect the activities of proteins that are involved in the proliferative response to mitogens, such as the cyclin D- and cyclin E-dependent protein kinases. Mutational analysis has identified a region of the c-*myc* coding region essential for the cotransformation, autoregulation and inhibition of differentiation functions of Myc (lying between amino acids 106-143)(Penn *et al*, 1990; Freytag, 1988; Stone *et al*, 1987), as well as regions involved with heterodimerisation, DNA binding and regions involved in transcriptional activation. These regions are also essential for apoptosis induced by c-*myc* (Evan *et al*, 1992).

Evan *et al* (1992) found that Rat-1 fibroblasts or primary rat embryo fibroblasts that constitutively expressed Myc were unable to undergo growth arrest (as measured by DNA synthesis) in low serum. However, the cell populations did not increase in number due to substantial apoptosis. Proliferating cells overexpressing c-*myc* were induced to undergo apoptosis by conditions causing cell cycle arrest (in G0 by serum deprivation, late G1 by isoleucine starvation or S by thymidine block). Apoptosis was dependent upon active Myc and upon regions of Myc required for transformation, autosuppression and inhibition of differentiation Further, cells arrested at various points in the cell cycle were induced to die upon induction of an oestrogen-regulable Myc (MycER). Therefore, the expression of c-*myc* brings with it at least two consequences: proliferation and susceptibility to apoptosis (Fig. 1.8).

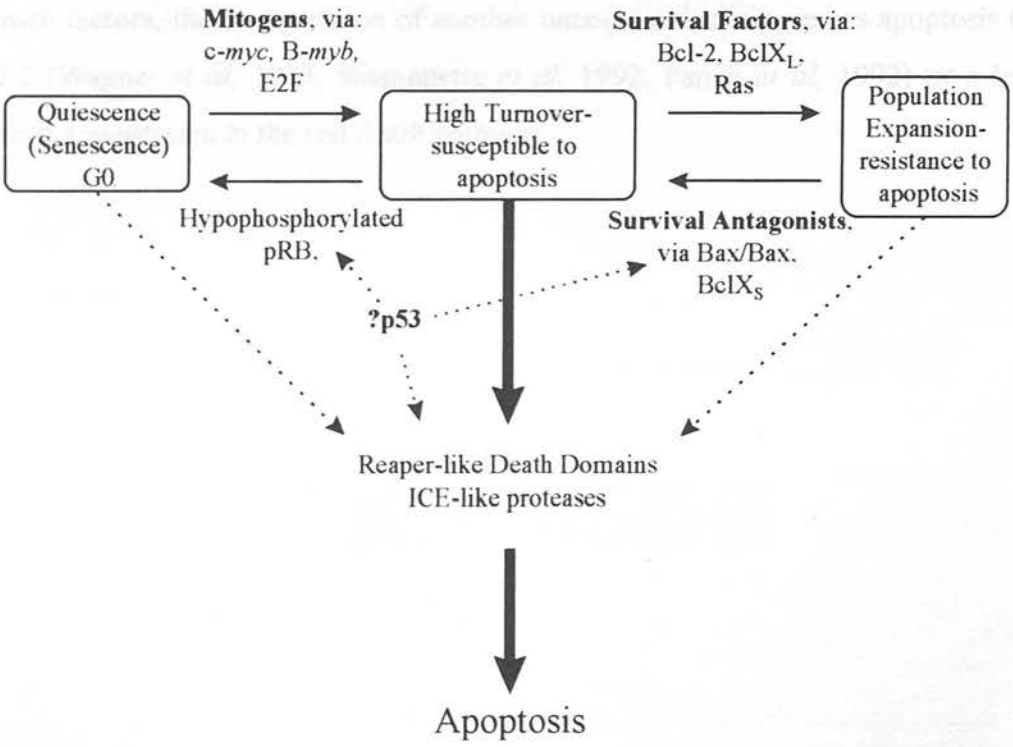
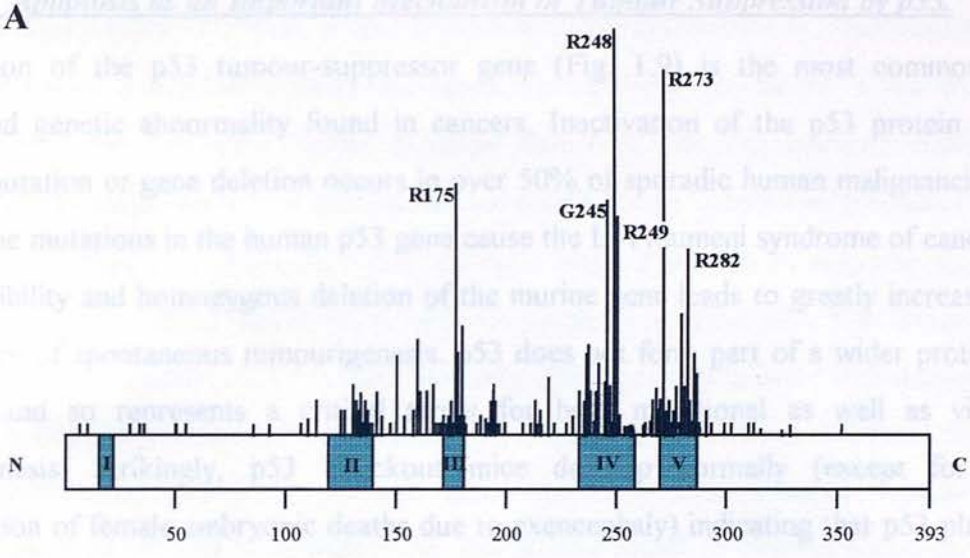


Fig. 1.8: Model for the Control of Apoptosis by Oncogenes such as c-*myc*. Activation of Mitogenic oncogenes such c-*myc* causes cells to enter a proliferative compartment where sensitivity to cell death caused by various stimuli is increased. This susceptibility can be altered by factors that affect survival and by wild-type p53.

As result of the requirement of Myc for proliferation, a proliferating cell is therefore primed to undergo apoptosis and will do so if there are simultaneous, conflicting signals urging it to both proliferate and arrest. In the case of deregulated expression of c-*myc*, a common occurrence in tumours of various types (Bishop, 1991), a fail-safe mechanism is therefore in place that deletes cells unless apoptosis is specifically inhibited. Rat-1 fibroblasts with deregulated Myc deprived of serum can be rescued from apoptosis by the addition of survival factors IGF-1 or PDGF which are unlinked to mitogenesis. (Harrington *et al*, 1994). The selective advantages provided by Myc in tumour cells need therefore to be reinforced either by continuous provision of growth factors, the co-operation of another oncogene that suppresses apoptosis (e.g. Bcl-2 (Wagner *et al*, 1993; Bissonnette *et al*, 1992; Fanidi *et al*, 1992) or a lesion further downstream in the cell death pathway.

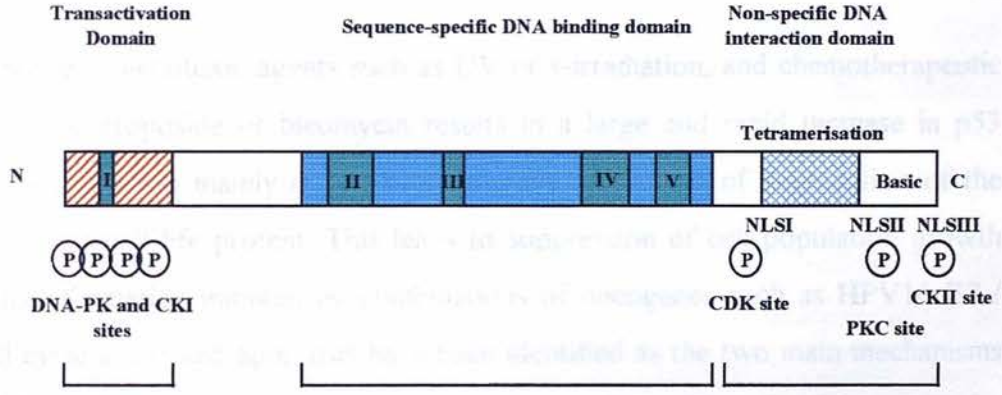
Fig. 1.9 (overleaf)**: The Structure of p53 protein.** (From Ko and Prives, 1996). A) Human cancer mutation frequency at various sites in the human p53 protein. Mutations cluster in the conserved regions (Roman numerals). Six of the most common 'hot-spot' mutations are labelled. B) Functional organisation of the p53 protein. P: phosphorylation sites. DNA-PK: DNA-dependent protein kinase. CK: Casein Kinase. CDK: Cyclin-dependent kinase. PKC: protein kinase C. NLS: Nuclear localisation signal. TAg: Large T antigen.

A



B

Functional Domains



Protein Interactions

TFIID
TBP
TAF40, TAF60
TFIIH
p62
MDM-2
RP-A
AdE1B-55k

SV40 TAg
p53BP1
p53BP2

TBP
TFIIH
XPB
XPD
CSB

1.1.5.6 Apoptosis as an Important Mechanism of Tumour Suppression by p53.

Alteration of the p53 tumour-suppressor gene (Fig. 1.9) is the most commonly observed genetic abnormality found in cancers. Inactivation of the p53 protein by point mutation or gene deletion occurs in over 50% of sporadic human malignancies. Germline mutations in the human p53 gene cause the Li-Fraumeni syndrome of cancer susceptibility and homozygous deletion of the murine gene leads to greatly increased incidence of spontaneous tumourigenesis. p53 does not form part of a wider protein family and so represents a critical target for both mutational as well as viral oncogenesis. Strikingly, p53 knockout mice develop normally (except for a proportion of female embryonic deaths due to exencephaly) indicating that p53 plays no essential role in normal cellular proliferation. On the other hand, the principle function of p53 is to mediate the cellular response to DNA damage, thereby maintaining genomic stability and preventing the accumulation of oncogenic mutations.

The response to genotoxic agents such as UV or γ-irradiation, and chemotherapeutic drugs such as etoposide or bleomycin results in a large and rapid increase in p53 protein concentration mainly owing to a decrease in the rate of degradation of the normally short half-life protein. This leads to suppression of cell population growth and of transformation initiated by combinations of oncogenes such as HPV16 E7 / *ras*. Cell cycle arrest and apoptosis have been identified as the two main mechanisms by which p53 exerts its oncosuppressive properties, although these may be supplemented by effects on differentiation, DNA repair, cellular senescence and angiogenesis. There has been much debate as to whether the cell cycle checkpoint activities of p53 are linked mechanistically to the apoptosis-promoting properties of p53. Therefore a discussion of research concerning both of the topics is relevant.

1.1.5.6.1 Control of the Cell Cycle by p53 (Fig. 1.10).

A number of inherited syndromes are associated with genetic instability and an increased susceptibility to cancer. One of these, ataxia telangiectasia (AT), an

autosomal recessive syndrome associated with progressive degeneration of cerebellar Purkinje cells leading to ataxia, is also complicated by radiosensitivity, radioresistant DNA synthesis and a measurably increased susceptibility to cancer. Cells from patients with this syndrome display a similar defect in their cell cycle checkpoint response to γ-irradiation to that of cells that have defects in p53 function. Kastan *et* al, (1992) discovered that AT cells failed toinduce wt p53 protein in response to γ-irradiation and that there was no delay of DNA synthesis in these cells. This indicates that the gene(s) mutated in AT is (are) normally upstream of p53 in a DNA-damage-cell-cycle-arrest signalling pathway. GADD45, a gene from a family of mammalian gene amplification and DNA damage genes inducible after ionising irradiation (Papthanasiou *et al,* 1991; Fornace *et al,* 1989), was found to contain a conserved p53-specific responsive element situated in its third intron. In contrast to normal cells, AT cells and cells without a functional wt p53 failed to induce GADD45 or p21 (see below) in response to γ-irradiation and failed to elicit cell cycle arrest (Artuso *et al,* 1995; Kastan *et al,* 1992). The designation of p53 as part of a pathway, lesions in any part of which leading to similar functional consequences, could explain how some cells sporting normal p53 genotypes may undergo PALA-selected gene amplification (Livingstone *et al,* 1992).

Overexpression of p53 in many cell types results in arrest of the cell cycle in late G1 although there is evidence to suggest that p53 may also play a role in arrests at G2 or mitosis (M). p53 can act as a sequence-specific transcriptional modulator, *trans*-activating some genes whilst repressing the expression of others. The ability of cells to undergo a growth arrest in response to DNA damage correlates well with ability of wild-type (wt) p53 and p53 point mutants to induce expression of a number of genes (Rowan *et al,* 1996; Crook *et al,* 1994; Pietenpol *et al,* 1994). The best characterised p53-responsive gene is $p21^{WAF1/CIP1}$ whose enhanced expression (by overexpression of p53 or by DNA damage) is responsible for inactivation of the cyclin / cyclin dependent kinase (cyclin/CDK) complexes that orchestrate the timing and the order of events of the cell cycle (Dulic *et al,* 1994; Xiong *et al,* 1993; Harper *et al,* 1993). p21, by binding via a C-terminal peptide domain, can also inhibit PCNA (an essential

processivity factor for DNA polymerases, including polymerase δ) (Nakanishi *et al*, 1995; Goubin and Ducommun, 1995; Luo *et al*, 1995; Chen *et al*, 1995; Pan *et al*, 1995) and thereby inhibits replicative DNA synthesis (Waga *et al*, 1994). Short gap filling by polymerases δ and ε is less sensitive to increased binding of PCNA by p21 and therefore nucleotide-excision repair of bulky DNA adducts is not as affected (Li *et al*, 1994). Deletion of the p21 gene results in complete abrogation of the p53-induced cell cycle arrest in HCT116 human colorectal carcinoma cells (Waldman *et al*, 1995). Inhibition of the G1 cyclins D and E by p21 prevents phosphorylation of the retinoblastoma gene product, pRB, thus quenching the transcriptional activity of E2F-1. In turn, E2F-1-responsive genes such as B-*myb*, which can bypass the effects of p21 overexpression (Lin *et al*, 1994), are therefore inactive: the cell arrests in late G1. A less complete effect of p21 deletion upon cell cycle arrest was seen in fibroblasts derived from p21 -/- mice (Bruarolas *et al*, 1995; Deng *et al*, 1995). This may reflect that other p53-induced proteins, such as GADD45 and cyclin G may also affect cell cycle progression and therefore represent redundant mechanisms of cell cycle control by p53.

1.1.5.6.2 Induction of p53-mediated Apoptosis and it's Relationship to Cell Cycle Control.

The first evidence that p53 was not only a cell cycle regulator but also had a role as an activator of cell death by apoptosis came in 1991 when Yonish-Rouach *et al* (1991) transfected a temperature-sensitive mutant of murine p53 (p53val135) into the M1 clone S6 mouse myeloid leukaemia cell line. This mutant was previously shown to induce a growth arrest in G1 in rat embryo fibroblasts at 32.5°C (Michalovitz *et al*, 1990). However, in the transfected M1 cells, a growth arrest was not measurable and cells rapidly underwent apoptosis when cells were shifted from 37.5°C to 32.5°C when the mutant p53 protein adopts the wild-type conformation (Yonish-Rouach *et al*, 1991). Apoptosis was greatest when cells were in the G1 phase of the cell cycle (Yonish-Rouach *et al*, 1993). In addition, colonic tumour-derived cells transfected

with a metallothionein-1 (MT-1) promoter-driven human wild-type p53 expression construct underwent increased apoptosis and regression of tumourigenesis in nude mice when induced with zinc chloride (Shaw *et al*, 1992). Similarly, apoptosis was induced at 32.5°C in murine erythroleukaemia (MEL) cells transfected with the p53val135 mutant (Ryan *et al*, 1993). The authors of this paper found that their cells arrested in G1 prior to undergoing apoptosis. Cells synchronised by density arrest and released into S phase before activation of the switch to wild-type p53 completed the current cell cycle before arresting in the next G1 before dying. Inhibition of cells at the G1/S boundary or in G0 by mimosine block or isoleucine starvation respectively did not result in cell death and it was concluded that p53-induced cell death and p53-mediated growth arrest are separate functions. Despite this, p21 expression was strongest in tissues and cells that have a high apoptotic rate (ElDeiry *et al*, 1994). Taken together, these studies suggested that p53-mediated apoptosis and G1 arrest was intimately but not necessarily mechanistically linked, although apoptosis induced by wild-type p53 upregulation occurs preferentially in the G1 phase of the cell cycle.

Analysis of tissues from mice with targeted knock-outs of their p53 alleles has definitively shown that p53 is required for DNA damage-induced apoptosis of thymocytes (Lowe *et al*, 1993; Clarke *et al*, 1993), lymphocytes (Howie *et al*, 1994), myeloid progenitor cells (Lotem and Sachs, 1993), and intestinal epithelium (Merritt *et al*, 1994; Clarke *et al*, 1994).

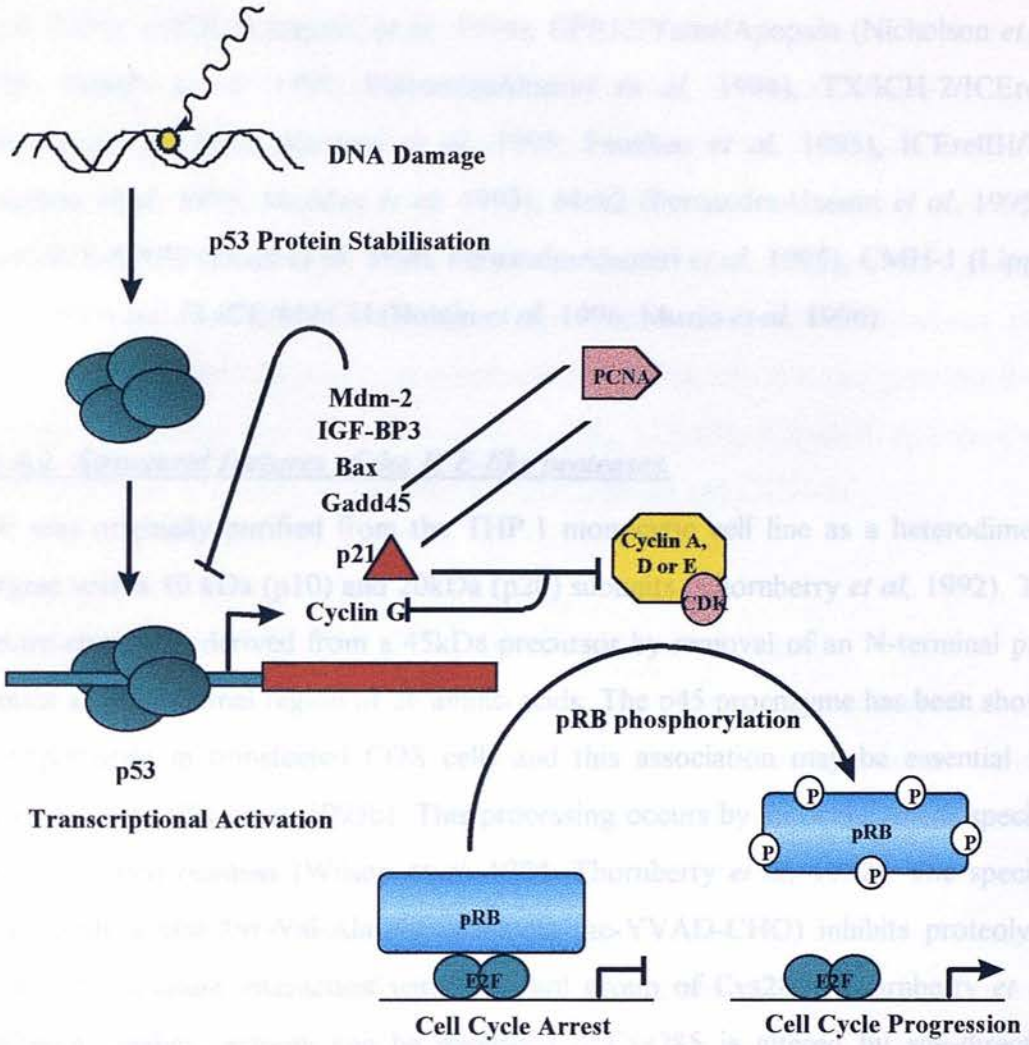


Fig. 1.10: DNA-Damage-Induced Growth Arrest Mediated by p53. (Based upon Bates and Vousden, 1996). DNA damage (e.g. ultraviolet light, γ-irradiation) is recognised and causes stabilisation of p53 which binds to recognition sites in various genes. Transcriptional activation causes increases in the concentration of several proteins, most importantly p21. p21 inhibits the activity of cyclin-dependent kinases resulting in hypophosphorylation of pRb. Hypophosphorylated pRb quenches the activity of the transcription factor E2F-1, which is essential for cell cycle progression into S phase.

the activities of other cysteine proteases of this novel family (the ICE-like proteases). To date the known family members include: ICH-1/Nedd-2 (Kumar *et al*, 1994; Wang *et al*, 1994), prICE (Lazebnik *et al*, 1994), CPP32/Yama/Apopain (Nicholson *et al*, 1995; Tewari *et al*, 1995; FernandesAlnemri *et al*, 1994), TX/ICH-2/ICErelII (Munday *et al*, 1995; Kamens *et al*, 1995; Faucheu *et al*, 1995), ICErelIII/TY (Faucheu *et al*, 1996; Munday *et al*, 1995), Mch2 (FernandesAlnemri *et al*, 1995a), Mch3/ICE-LAP3 (Duan *et al*, 1996; FernandesAlnemri *et al*, 1995), CMH-1 (Lippke *et al*, 1996) and FLICE/MACH (Boldin *et al*, 1996; Muzio *et al*, 1996).

1.1.6.1 Structural features of the ICE-like proteases.

ICE was originally purified from the THP.1 monocytic cell line as a heterodimeric enzyme with a 10 kDa (p10) and 20kDa (p20) subunits (Thornberry *et al*, 1992). The mature enzyme is derived from a 45kDa precursor by removal of an N-terminal pro-domain and an internal region of 26 amino acids. The p45 proenzyme has been shown to oligomerise in transfected COS cells and this association may be essential for autoprocessing (Gu *et al*, 1995b). This processing occurs by autocatalysis at specific aspartate (Asp) residues (Wilson *et al*, 1994; Thornberry *et al*, 1992). The specific tetrapeptide acetyl-Tyr-Val-Ala-Asp-aldehyde (ac-YVAD-CHO) inhibits proteolysis by ICE by covalent interaction with the thiol group of Cys285 (Thornberry *et al*, 1992)and catalytic activity can be abolished if Cys285 is altered by site-directed mutagenesis. Thus ICE was classified as a cysteine protease with substrate specificity for Asp at the P1 position (the first amino acid N-terminal to the cleavage site). Although the active cysteine is located in the p20 subunit, X-ray crystallography has shown that other critical amino acids concerned with the formation of the substrate binding pocket are to be found in both the p10 and p20 subunits. These structural studies also suggest that two p10/p20 heterodimers form a tetrameric active enzyme complex (Walker *et al*, 1994; Wilson *et al*, 1994; Thornberry *et al*, 1992).

Sequence comparison between ICE and other family members show approximately 30% identity and suggest synthesis of inactive proenzymes is a common feature of all these proteases. The amino acids involved in recognition of the P1 Asp as well as the

QACRG sequence containing the active cysteine are conserved in all members. More diversity is found in the amino acid residues lining the groove for the P2-P4 substrate positions and this may explain differences in the substrate preferences of the individual family members. While subunit molecular masses have only been accurately determined for ICE, CPP32 (Nicholson *et al,* 1995) and Ced-3 (Xue *et al,* 1996), it seems likely from sequence data and the available X-ray crystallography (Rotonda *et al,* 1996) that all the family members are proteolytically processed into a larger and a smaller subunit and that these form active heterotetrameric enzyme complexes. From the predicted size of the proenzyme and active subunits, the ICE-like protease family has been subdivided into the ICE-subfamily (ICE, ICErelII, ICErelIII) and the Ced-3 subfamily (Ced-3, CPP32, ICH-1/Nedd2, Mch2, Mch3 and CMH-1).

1.1.6.2 Evidence for the Role of ICE-like Proteases in Apoptosis.

Overexpression of any of the proteases in Rat1 cells results in apoptosis. However, this test alone is not sufficient to prove that a protease is directly involved with the apoptotic program. It has been claimed that loading of cells with proteases with entirely different substrate specificities, such as trypsin and proteinase K, is capable of inducing cell death that displays some of features of apoptosis including membrane blebbing, chromatin condensation and DNA degradation (Williams and Henkart, 1994). Conclusive evidence for a role of the ICE-like proteases in apoptosis has come from experiments using natural or synthetic protease inhibitors, knockout mice and the characterisation of protease activity in apoptotic cell extracts.

1.1.6.2.1 Viral Inhibitor Proteins.

The existence of viral proteins that act as inhibitors of ICE-like proteases is indicative of the fact that apoptosis is a significant factor in the defence against viral infection. Expression of the cowpox virus gene *crmA* suppressed apoptosis induced by overexpression of ICE in Rat1 cells (Miura *et al,* 1993). CrmA is also capable of inhibiting apoptosis induced by growth factor withdrawal (Li *et al,* 1996; Gagliardini *et al,* 1994), anti-Fas (Tewari *et al,* 1995b; Tewari and Dixit, 1995b; Los *et al,*

1995b) and TNFα (Hsu *et al*, 1995). Some authors have noted that while CrmA can inhibit apoptosis induced by Fas or activation in lymphoid cells, it is less capable of inhibiting apoptosis induced by other stimuli such as dexamethasone (Memon *et al*, 1995), γ-irradiation or serum starvation (Strasser *et al*, 1995). The explanation for this may lie in the observation that CrmA fully inhibited ICE at 10^4 fold less concentration than that required to inhibit CPP32 (Nicholson *et al*, 1995). It is known that Fas-mediated, but not dexamethasone- or γ-irradiation-induced apoptosis, is abolished in thymocytes from ICE knockout mice (Kuida *et al*, 1995).

A baculovirus protein, p35 inhibits premature apoptosis of infected insect cells thus increasing the efficiency of virus production (Clem and Miller, 1994; Crook *et al*, 1993). p35 is capable of inhibiting cell death of cells in embryonic (and *reaper* transgenic) *Drosophila* and *C. elegans* (Hay *et al*, 1994; Sugimoto *et al*, 1994; White *et al*, 1994) and blocks the activity of the mammalian proteins ICE, CPP32, ICH-1 and ICH-2 (Bump *et al*, 1995). It seems probable that p35 has a broader range of target proteases than CrmA. Both p35 and CrmA have Asp residues in the context of ICE-like protease cleavage sites and act as suicide substrates for those enzymes: cleaved inhibitor protein is less easily dissociable from the enzyme than an ordinary substrate and therefore acts as an efficient inhibitor. Intriguingly, the broad spectrum of targets enjoyed by p35 can be transferred to CrmA by an exchange of protease cleavage sites (Xue and Horvitz, 1995) indicating that these two viral proteins act on specific ICE-like proteases by very similar molecular mechanisms.

1.1.6.2.2 Synthetic Protease Inhibitors.

Small peptides corresponding to the protease cleavage site can act as substrates and compete with endogenous substrates in cells or cell extracts. If the carboxy terminus of the peptide is derivatised at the P1 position with aldehyde or halomethylketone groups, these small peptides become extremely efficient inhibitors of protease activity and substrates with fluorogenic derivatives can be used to monitor enzyme activity (Pennington and Thornberry, 1994; Thornberry and Molineaux, 1995). In addition,

chloro- or fluoromethyketone derivatives also confer the advantage that the peptide is permeable to cell membranes allowing inhibition to occur in intact cells. The peptide ac-YVHD-CHO, corresponding to the ICE cleavage site in proIL-1β, is capable of inhibiting ICE. Substitution at the P2 position is tolerated and the peptide YVAD is the most potent ICE inhibitor peptide in practice and was used to purify ICE to homogeneity from cell extracts (Thornberry *et al,* 1992). Derivatives of YVAD that inhibit ICE activity have been shown to inhibit, for example, apoptosis mediated by Fas (Los *et al,* 1995; Enari *et al,* 1995), *reaper* overexpression in *Drosophila* (Pronk *et al,* 1996) or trophic factor-deprived chicken motor neurones (Milligan *et al,* 1995).

In a similar way to that described for CrmA, apoptosis induced by other means cannot always be inhibited by YVAD peptides at concentrations at which cleavage of proIl-1β is completely inhibited (Nett-Fiordalisi *et al,* 1995). Although YVAD is capable of inhibiting the activity of all of the ICE-like proteases, many of the enzymes require a much higher concentration of YVAD than does ICE to achieve complete inhibition. This may be explained by differences in substrate preference for different ICE-like proteases. Whereas ICE cannot cleave PARP (see below) except at high substrate concentrations, peptides that include the DEVD sequence inhibit PARP cleavage and apoptosis induced by enzymes such as CPP32 which have a strong preference for that sequence. Indeed CPP32 has been purified to homogeneity using DEVD peptides from the same cell line that ICE was similarly purified using YVAD peptides. (Nicholson *et al,* 1995). This difference in substrate preference between different members of the same enzyme class may suggest that each member may perform different functions in the apoptotic program or that they are differentially utilised depending upon the source of the apoptotic stimulus. However, although some of these patterns can be discerned, the fine detail is unlikely to be completely defined using peptide inhibitors alone because of the incomplete specificity of the enzymes for any one peptide substrate. The combinatorial analysis of ICE-like protease gene knockout mice may resolve this.

1.1.6.3 Target Substrates of the ICE-like Proteases.

A large number of proteins are cleaved into specific fragments during apoptosis. One of the first identified substrates of an ICE-like protease was NAD^+-dependent poly(ADP-ribose)polymerase (PARP). PARP is activated by double strand DNA breaks and adds chains of ADP-ribose to many proteins, including histones and DNA ligase II. Early on in apoptosis, PARP is inactivated by protease cleavage at an Asp residue which separates the DNA-binding domain from the catalytic domain. It is thought that this inactivation of PARP conserves energy, thus allowing apoptosis (a process that requires ATP) to proceed to completion (Earnshaw, 1995; Kaufmann *et al*, 1993; Althaus and Richter, 1987).

A number of candidates for the PARP-cleavage enzyme have been proposed. A protease with similar preference for Asp residues at the P1 position as ICE (prICE) was identified in chicken cell apoptotic extracts. As this protease activity could not process proIL-1β (Lazebnik *et al*, 1993) and as ICE needed to be present at 50-100 fold higher concentration to cleave PARP than to process proIL-1β in a transient overexpression assay (Gu *et al*, 1995a), it is thought that the PARP cleavage enzyme is not ICE. On the other hand, recombinant CPP32 can cleave purified PARP *in vitro* (Tewari *et al*, 1995b). When overexpressed, CPP32 cleaved PARP in stable PARP-transfected cell lines (FernandesAlnemri *et al*, 1994) and CPP32 was activated at the same time that PARP cleavage occurred in anti-Fas-treated Jurkat cells (Schlegel *et al*, 1996). Cleavage of PARP can be inhibited by DEVD peptides (Schlegel *et al*, 1996; Nicholson, 1996) and other members of the ICE-like protease family, including Ced-3, ICErelII, Nedd2, Cmh-1, Mch2 and Mch3, are all capable of cleaving PARP (FernandesAlnemri *et al*, 1995a; Duan *et al*, 1996a; Xue and Horvitz, 1995a; Lippke *et al*, 1996a; Gu *et al*, 1995a). CPP32 and its close relations, Mch3 and Cmh-1 which also have similar enzyme kinetics to CPP32, are the most likely candidate PARP cleavage enzymes.

Addition of purified CPP32 to isolated nuclei is not sufficient to cause changes in nuclear morphology (Nicholson *et al*, 1995) and PARP knockout mice do not show

the apoptotic proteases work in concert with each other to cause cell death and that substrates other than PARP are responsible for the changes in cellular morphology. Importantly, some components of the cytoskeleton and proteins that control cell shape such as fodrin, G-actin and Gas2 are known to be cleaved during apoptosis by as yet unidentified proteases (Kayalar *et al,* 1996; Brancolini *et al,* 1995; Martin *et al,* 1995). The adenomatous polyposis coli protein (APC) which is associated with junctional complexes is also cleaved during apoptosis (Browne *et al,* 1994).

In the nuclear envelope, intermediate filament proteins known as the nuclear lamins are probably responsible for maintenance of the integrity of the nuclear membrane and these proteins are cleaved in apoptosis (Lazebnik *et al,* 1993). In contrast to the early, parallel degradation of proteins such as PARP and U1 ribonuclear protein, lamin proteolysis occurs from 10 minutes following the completion of PARP cleavage. This late proteolytic step is 100 times more sensitive to YVAD than PARP and addition of the chemical inhibitor TLCK to apoptotic cell extracts inhibits the formation of nuclear fragments whilst allowing DNA fragmentation and chromatin condensation to occur (Lazebnik *et al,* 1995a; Lazebnik *et al,* 1995b). Therefore, events in the apoptotic program are temporally controlled with activation of a protease (probably CPP32) marked by PARP cleavage. This is followed by lamin proteolysis (most likely by Mch2 and not CPP32 (Takahashi and Earnshaw, 1996)) at a later stage which results in the break-up of the nucleus into membrane bound fragments. These data suggest that a central proteolytic cascade of ICE-like proteases controls events in the apoptotic program in an ordered fashion.

There is further evidence for a cascade-type architecture in which the ICE-like proteases play a central role. Overexpression of the protease precursors in bacteria or eukaryotic cells can lead to autocatalytic activation. CPP32, however, can be activated by other ICE subfamily proteases (Enari *et al,* 1996; Shimizu *et al,* 1996; Tewari *et al,* 1995). Likewise ICErelII can cause processing of ICE precursor in COS cells and Mch3 contains a DSVD sequence that is similar to the CPP32 cleavage site. In addition, thymocyte apoptosis induced by several agents can be halted at various

stages with protease inhibitors of different specificities (Zhivotovsky *et al,* 1995; Fearnhead *et al,* 1995). Therefore, various cell death stimuli converge on the same molecular pathway that results in apoptosis.

1.1.6.4 Regulation of the Protease Cascade (Fig.1.11).

Once activated, the ICE-like proteases act as a co-ordinated engine that drives apoptosis in a stereotyped and temporally controlled manner. The internal organisation of this central pathway has been discussed above: the substrates for each protease include other members of the ICE-like protease family that act at a lower level in a protease cascade.This type of organisation could therefore ensure that the morphological and other biochemical events of apoptosis occur in the correct order and, depending on the enzyme kinetics for each substrate at each level, could also regulate the timing of events. Despite the recognition of some of the components of this death mechanism and their likely interactions, the molecular mechanism by which death and survival signals are integrated into the decision of a cell to die, or how these decisions activate the ICE-like protease cascade, is not known. A number of observations outlined below illustrate potential control points.

At the transcriptional level, all of the ICE-like protease mRNAs can be detected in most tissues. One mechanism of activation of the proteases may be to cause an increase in the intracellular concentration of one or more of the enzymes. To support this, ICE is induced by loss of cellular adhesion and by the transcription factor IRF-1 (Tamura *et al,* 1995; Boudreau *et al,* 1995). mRNA splice variants have been described for some of the family members including ICE, ICH-1/Nedd-2, Mch2 and Mch3 (FernandesAlnemri *et al,* 1995b; Wang *et al,* 1994b; FernandesAlnemri *et al,* 1995b; Alnemri *et al,* 1995b). The shorter of the two human ICH-1 splice variants encodes a truncated form of the protein (ICH-1_S) that does not contain the active QACRG sequence. Expression of ICH-1s in Rat-1 cells inhibited apoptosis induced by ICH-1_L or serum deprivation presumably by hetero-oligomerisation between ICH-1s and ICH-1_L (Wang *et al,* 1994). However, the mouse equivalent, Nedd2_S, has no activity towards cell death in FDC-p1 cells (Kumar and Harvey, 1995). Hetero-

oligomerisation between members of the same sub-family, for example between CPP32 and Mch3 or between ICE and ICErelII, has been observed (Gu *et al,* 1995b; Faucheu *et al,* 1995b; FernandesAlnemri *et al,* 1995b) and this may act to regulate enzyme activity or to alter substrate preferences.

Post-translational modification of the precursor protein seems the most likely control point for the ICE-like proteases. A role for glycosylation or phosphorylation has not been shown. Therefore, the critical step appears to be at the point of cleavage of the first protease precursor in the cascade into its active subunits. In the Fas death pathway, ligation of CD95/Fas by its ligand results in the activation of the CPP32-like activity of FLICE/MACH by direct protein-protein interactions at the cytoplasmic death domain of the receptor (Muzio *et al,* 1996; Boldin *et al,* 1996). FLICE and CPP32 can also be directly activated by proteolysis by the CTL granule serine protease Granzyme B (Muzio *et al,* 1996; Martin *et al,* 1996; Quan *et al,* 1996; Darmon *et al,* 1995).

Not all death pathways are so simple, however, as some death signals may not always result in apoptosis. It is the integration of death and survival signals that determines the activation of the ICE-like proteases in these situations. The mechanisms to deal with these conflicting signals are therefore likely to be more complex than in Fas-mediated apoptosis. While activation of the proteases is not well understood, negative regulators of apoptosis have received much attention. Members of the Bcl-2 family are related to the *C. elegans ced*-9 gene that negatively regulates the activity of *ced*-3 and *ced*-4 in embryonic cell death. Bcl-2, $BclX_L$ and viral homologues such as adenovirus E1B-19K have been shown to block cell death by many stimuli (see above). Activation of CPP32 is dependent upon the activation of ICE in PC12 cells and activation of ICE is blocked by Bcl-2 or $BclX_L$ (Shimizu *et al,* 1996). Similarly activation of CPP32 or Mch3 by E1A or following treatment with staurosporine was blocked by Bcl-2 or its apoptosis inhibitory homologues (Boulakia *et al,* 1996; Chinnaiyan *et al,* 1996). In contrast, Fas- or TNFR-1-mediated apoptosis, while sensitive to inhibition of protease activity by CrmA, is not sensitive to Bcl-2 or

$Bcl X_L$ (except in the liver)(Duan *et al,* 1996; Memon *et al,* 1995; Chiu *et al,* 1995; Rodriguez *et al,* 1996), indicating that the point of control of most apoptotic pathways by *ced*-9 homologues lies upstream, or at the activation step of the ICE-like proteases.

1.1.7 Apoptosis: Remaining Questions.

As is apparent from the discussion above, there are many aspects of the control and execution of the apoptosis pathway that remain to be worked out despite intensive research activity that has led, in only a few years, to a recognition of the main molecules involved. In particular, the precise mechanisms by which molecules such as p53 and c-*myc* transmit death decisions to effector molecules (ICE-like proteases) remains to be determined. Co-operation between p53 and c-*myc* in the induction of apoptosis has been hypothesised. Experiments where apoptosis could be triggered by specific manipulation of the expression of individual (or combinations of) genes would be most informative, especially in stable cell lines or transgenic animals. Such systems could be eminently suitable for biochemical analysis and genetic dissection of events downstream of the transfected gene.

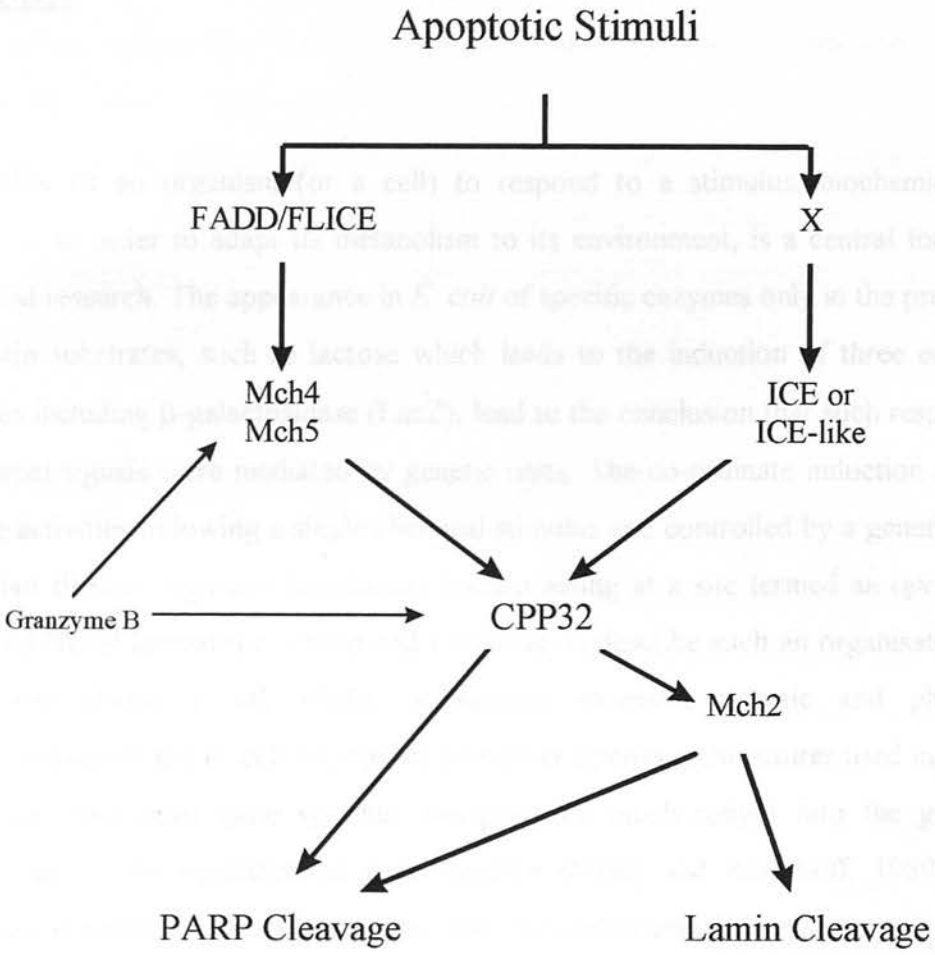


Fig. 1.11: Organisation of the ICE-like Protease Cascade. (Adapted from Fernandes-Alnemri *et al*, 1996). This scheme outlines the known proteolytic events that control the order and timing of the apoptosis effector program. A stimulus (FasL, Granzyme B or other stimulus X) results in activation of ICE-like proteases culminating in the activation of similar enzymes such as CPP32 that can mediate end-substrate cleavage or activation of downstream proteases that can cleave a different range of substrates.

1.2 Introduction: Conditional Mammalian Expression Systems.

The ability of an organism (or a cell) to respond to a stimulus, biochemical or otherwise, in order to adapt its metabolism to its environment, is a central focus of biological research. The appearance in *E. coli* of specific enzymes only in the presence of certain substrates, such as lactose which leads to the induction of three enzyme activities including β-galactosidase (LacZ), lead to the conclusion that such responses to external signals were mediated by genetic units. The co-ordinate induction of the enzyme activities following a single chemical stimulus and controlled by a genetically-linked but distinct regulator (repressor) protein acting at a site termed an *operator*, prompted Nobel laureates F. Jacob and J. Monod to describe such an organisation as an *operon* (Jacob *et al*, 1960). Subsequent extensive genetic and physical characterisation of the E. coli *lac* operon as well as operon architectures used in other prokaryotic and yeast gene systems, has provided much insight into the general mechanisms of the regulation of gene function (Miller and Reznikoff, 1980), the principles of which can be applied to the more complex mammalian systems.

This project focused upon the application of experimental gene regulation systems for use in mammalian cells such that the activity of specific, introduced proteins could be specifically controlled in a temporal manner. In particular, genes known to have negative effects on cell viability or cell cycle were chosen for study for two reasons: Firstly, p53, c-*myc* and *nedd*2 as discussed above, have previously been directly linked to effects on apoptosis and (or) growth arrest and these properties may affect pathological processes such as oncogenesis. In addition, $p21^{WAF1\text{-}CIP1}$ has been proposed as a candidate mediator of the oncosuppressive activity of p53. The ability to experimentally regulate the expression of these genes is clearly of interest to researchers working in the related fields of cancer biology and apoptosis. Secondly,

because of their potential to cause cell death or prolonged growth inhibition, p53, p21 and *nedd2* are reasonable test genes to evaluate the stringency of regulation of gene expression in experimentally inducible gene expression systems. The originators of many of these systems have made suggestions, based upon relatively lower levels of expression of reporter genes in the uninduced state, that such systems should be suitable for the controlled expression of deleterious genes in tissue culture or transgenic animals. While such systems have been used to regulate expression of genes that positively control cell growth or are at least neutral as regards cell death and cell cycle effects, confirmation of such suppositions for genes that have intrinsic death-inducing activity has been lacking in the literature.

1.2.1 Constitutive versus Conditional Gene Expression.

A significant problem when investigating the functions of genes putatively involved in the control of apoptosis is the difficulty of producing stable cell lines that show elevated levels of expression of a cell death transgene. Conventional expression vectors containing apoptosis genes, such as *Ich1 Nedd* (Wang *et al*, 1994), which cause constitutive overexpression, can only be used effectively in transient expression assays. This sort of assay usually requires the cotransfection of, or fusion with a reporter gene such as *lacZ* in order to mark transfected cells. If the test gene is lethal, then the majority of *lacZ*-positive cells will be apoptotic (Miura *et al*, 1993). The value of transient transfection assays in dissecting biochemical pathways is limited as the transfection efficiency is often low and there is considerable heterogeneity in both level and timing of expression. Furthermore, it has not been possible to generate transgenic animal strains in which the *in vivo* effects of cell death gene expression can be studied. An exception to this is the use of constitutive promoter/enhancer elements that are conditional in the sense that they are active only in specific tissues.

An alternative approach for expression of cloned apoptosis genes is to use inducible genes. The activity of these genes should ideally 1) be specifically, rapidly and reversibly inducible by exogenous stimuli that otherwise have no detectable biological

activity in the test system; and 2) show minimal expression in the uninduced state, but good induction in the presence of an inducing stimulus. The remaining portion of this chapter introduces the gene systems that were considered for use in this project. Firstly, exogenous control at the protein level is considered for a temperature-sensitive allele of murine p53 and for fusion of steroid receptor hormone-binding domains to oncoproteins such as c-Myc. A survey of inducible promoter systems derived from mammalian viruses or endogenous mammalian genes follows. Finally, synthetic inducible vector systems derived from components drawn from E.coli, yeast and mammalian DNA and that have been claimed to conform closely to ideals 1) and 2) above are discussed.

1.2.2 Temperature-Sensitive Mutant p53

Gannon *et al*, (1990) defined two alternative conformations of the p53 protein product: a pseudo-wild-type, growth-suppressor product immunoreactive with the monoclonal antibody PAb246 (Cook and Milner, 1990; Yewdell *et al*, 1986; Wade-Evans and Jenkins, 1985) and "an overtly mutant ($PAb240^{+}$) form" (Gannon *et al*, 1990). It appears that a variety of activating mutations in p53 cause a common conformational shift (Gannon *et al*, 1990; Milner and Cook, 1986). Further, the conformation of temperature-sensitive mutants, for example murine p53Val135, can be switched between a $PAb246^{+}$ form (pseudo-wild-type) at 32°C to an activated mutant conformation (PAb240 reactive, $PAb246^{-}$) within 5 minutes upon temperature shift to 37°C (Milner and Medcalf, 1990).

Whilst wt-wt and mutant-mutant p53 complexes form readily when mixed post-translationally in vitro, complexes containing mutant and wt p53 can only be produced when translated together (Milner *et al*, 1991). Using the temperature-sensitive mutant p53val135 and a cell-free translation system, Milner and Medcalf, (Milner and Medcalf, 1990) showed that the product of a wild-type allele is forced into the mutant

conformation when co-translated with temperature sensitive mutant p53 at 37°C (Fig. 1.12). Further, when translated at 32°C the complex formed consisted exclusively of p53 molecules in the wild-type ($PAb246^+$, $PAb240^-$) conformation. Upon temperature shift to 37°C there was a loss of reactivity of PAb246 and a co-ordinate gain in PAb240 reactivity indicating that the mutated p53 had driven the product of the wild-type allele into the mutant form. This shift can occur during or after translation (Fig. 1.12).

In parallel to a temperature-dependent shift in antibody reactivity, p53val153 also undergoes a shift in function. When overexpressed in some cell lines, the temperature-sensitive mutant acts like wild-type p53 at 32°C by causing apoptosis (Yonish-Rouach *et al*, 1991) and (or) growth arrest in late G1 (Ryan *et al*, 1993; Yonish-Rouach *et al*, 1993; Ginsberg *et al*, 1991; Michalovitz *et al*, 1990). At 37°C, overexpressed p53val135 has the properties of a dominant negative mutant p53. Therefore this mutant can be used to study the overexpression of both mutant and wild-type p53 in a single stably-transfected cell line by maintenance of cells in culture at 37°C until a temperature shift to 32°C (wild-type) is required.

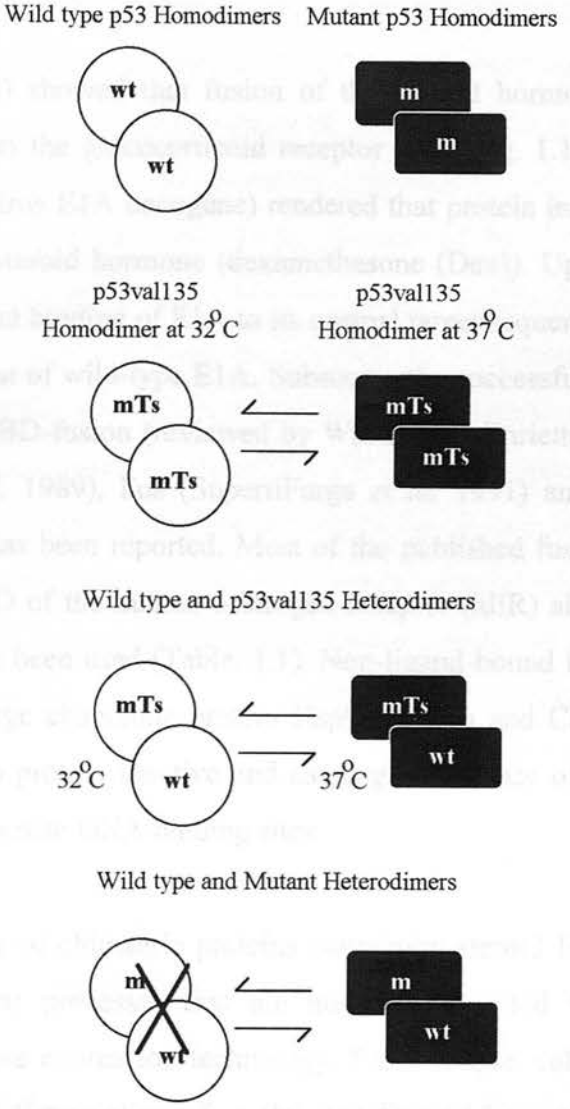


Fig. 1.12: Conformational Model of the Dominant-negative Effect of Mutant p53. p53 commonly oligomerises. Wild type (wt) only complexes adopt one conformation (circles) whereas mutant (m) homo-oligomers adopt a distinct conformation (rectangles). Temperature-sensitive p53 (p53val135) (mTs) homo-oligomers can adopt either conformation depending on the temperature and readily change conformation upon temperature shift. Many p53 mutants including p53val135 at 37°C can force wt p53 to adopt the mutant conformation when co-translated. The conformational influence upon wt p53 by p53val135 is reversed upon temperature shift to 32°C. Complexes containing non-temperature-sensitive mutant p53 molecules can adopt only one conformation (whereas wt only complexes may adopt a mutant-like conformation upon DNA binding (Halazonetis *et al*, 1993)).

1.2.3 Steroid Hormone-Regulable Chimaeric Proteins

Picard et al, (1988) showed that fusion of the steroid hormone receptor binding domain (HBD) from the glucocorticoid receptor (GR)(Fig. 1.13) to a heterologous protein (the adenovirus E1A oncogene) rendered that protein inactive in the absence of the appropriate steroid hormone (dexamethasone (Dex)). Upon addition of Dex, however, activity and binding of E1A to its normal target sequence (E3) was restored to levels close to that of wild-type E1A. Subsequently, successful regulation of many other proteins by HBD-fusion (reviewed by Walker and Enrietto, (1995)), including c-Myc (Eilers *et al,* 1989), Fos (SupertiFurga *et al,* 1991) and p53 (Roemer and Friedmann, 1993) has been reported. Most of the published fusion proteins involve fusion with the HBD of the human oestrogen receptor (hER) although other steroid receptor HBDs have been used (Table. 1.1). Non-ligand-bound HBDs are thought to interact with the large chaperone protein Hsp90 (Green and Chambon, 1991) thus rendering the fusion protein inactive and causing the release of fused transcription factors from their cognate DNA binding sites.

The regulable nature of chimaeric proteins containing steroid HBDs has facilitated the study of cellular processes that are not ideally suited to dissection using traditional constitutive expression technology. For example, cell culture conditions that determine the differentiation of v-Rel transformed bone marrow cells to two independent cell lineages were arrived at using cell clones derived by transformation with a v-Rel-ER vector (Boehmelt *et al,* 1995). Discrimination between the short- and long-term effects of c-Fos expression on the differentiation of polarised epithelial cells was achieved with a Fos-ER construct. This technology also allows a distinction to be drawn between the proximal effects of proto-oncogene overexpression that may initiate cellular proliferation from irreversible changes that occur later in the course of oncogene-driven transformation. In addition, HBD fusion to proteins that themselves activate differentiation programs (e.g. C/EBP (Umek *et al,* 1991)) or induce cell death (e.g. p53 (Roemer and Friedmann, 1993)) allow the transfected cells to be propagated in the absence of inducer until stimulated with ligand. Fusion

specific recombination proteins, such as Cre and FLP, have enabled the design of increasingly more complex and biologically meaningful gene targeting experiments that overcome the problems of embryonic lethality of many mutations inserted by conventional targeting technology (Zhang *et al*, 1996; Metzger *et al*, 1995; Logie and Stewart, 1995). The c-Myc-ER fusion protein was instrumental in the demonstration that constitutive activation of c-*myc* in serum-deprived Rat-1 fibroblasts not only caused inappropriate cell proliferation but also induced apoptosis (Evan *et al*, 1992) that was rescued by specific survival factors such IGF-1 (Harrington *et al*, 1994). Owing to the low toxicity and specificity of the inducing agents, the steroid receptor chimaeras, in particular those involving the ER HBD, together with some other inducible gene systems, open up the possibility of identification of downstream target genes of transcription factors by subtractive or differential cDNA techniques from otherwise identical cell lines or tissues. Using these methods, MHC-I and HMG14(b) were two genes whose expression were altered by ligand-activated v-relER (Boehmelt *et al*, 1992).

The use of fusion proteins has several advantages over the isolation and use of temperature-sensitive mutants. Firstly, it is often more convenient to construct a fusion gene *in vitro* than it is to screen many potential mutant proteins for temperature-sensitive activity *in vivo*. Secondly, fusion protein activity is tightly controlled by the presence or absence of ligand and switching of activity is fast and readily reversible. Often, ts proteins, for example p53val135, although readily reversible by temperature shift are not completely 'on' or 'off' at a particular temperature and may need to be shifted to temperatures that are non-physiological (and that may elicit the pleiotropic heat-shock response) in order to achieve maximal effect. However, use of fusion proteins have a few disadvantages: Responses to ligand cannot be studied in cells with endogenous hormone receptors or that produce endogenous ligand. In most cases, though, the HBD chosen is an attenuated human hER (HE14: Kumar *et al*, (1986)) which has a mutation (G400V) that results in relative insensitivity to endogenous mildly oestrogenic compounds but not to the physiological inducer: 17β-oestradiol. In addition, laborious procedures are required

to charcoal-strip steroids from serum used in tissue culture and cells must be cultured in medium devoid of Phenol Red, a mildly oestrogenic pH indicator dye. This procedure may not completely prevent low-level activation of the hER however (Berry *et al*, 1990; Danielian *et al*, 1993). It is possible that oestrogenic compounds may also leach from certain tissue culture plastics and thus confound experiments with ER-fusion proteins. Also, when considering experiments with transcription factor-HBD fusions, it is important to exclude the possibility that, other than the introduction of steroid sensitivity, fusion of the receptor to an heterologous protein does not itself produce a novel activity. Disruption of important protein functions caused by an adjacent HBD fusion can be controlled for by making two fusion proteins: fusions being made for both the amino or carboxyl termini. Further, HBDs of steroid receptors overlap with a region that contain endogenous transcriptional activation property (the TAF-2 activity). The TAF-2 activity may contribute to ligand-independent activity: for example in a FosB-ER fusion (Schuermann *et al*, 1993). The transcriptional activity of ER TAF-2 can be separated from the activity of the fused transcription factor by comparison of the effects of ligand,17β-oestradiol, and its analogues ICI 164.384 and Z-4-hydroxytamoxifen (4-OHT). ICI164.384 [N-n-butyl-11(3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl)-N-methylundecamide] (Wakeling and Bowler, 1988) is a competitive antagonist of the oestrogen receptor and its TAF-2 activity and therefore results in complete inactivation of fusion proteins. However, 4-OHT is a partial antagonist that, like 17β-oestradiol stimulates the TAF-1 activity in the amino terminus and release of Hsp90 by the ER. 4-OHT does not, however, stimulate the TAF-2 activity. Therefore 4-hydroxytamoxifen activates ER-fusion proteins but does not elicit transactivation by TAF-2 in such a setting.

Many of the drawbacks of the ER HBD domain in fusion proteins has been recently overcome by the isolation of a mutant murine ER (G525R: ER^{tm}) that is insensitive to estradiol but remains sensitive to 4-hydroxytamoxifen (Littlewood *et al*, 1995; Danielian *et al*, 1993). The G525R mutant disrupts the TAF-2 activity and alleviates the need to use charcoal-stripped serum and Phenol Red-free medium. It also opens up the possibility of using ER^{tm}-fusion proteins as transgenes *in vivo*. Tamoxifen has

been well characterised in both animal and human trials, being metabolised to 4-hydroxytamoxifen in the liver, although it has been shown to exert oestrogenic effects (probably mediated by TAF-1-dependent *trans*-activation) upon certain tissues such as endometrium and bone (Gottardis *et al,* 1988; Jordan *et al,* 1987; Jordan, 1984).

Table. 1.1. Steroid receptors used in chimaeric proteins (Walker and Enrietto, 1995)

Steroid Receptor	Ligand	Action of Ligand
Glucocorticoid Receptor (GR)	Dexamethasone	Agonist
	Progesterone	Antagonist
	RU486	Antagonist
Oestrogen Receptor (ER)	17β-oestradiol	Agonist
	Diethylstilbesterol	Agonist
	Tamoxifen	Partial Agonist
	ICI 164.389	Antagonist
Mineralocorticoid Receptor (MR)	Aldosterone	Agonist
	Spironolactone	Antagonist
Drosophila Ecdysterone Receptor (EcR)	Ponasterone A	Agonist
	Muristerone A	Agonist

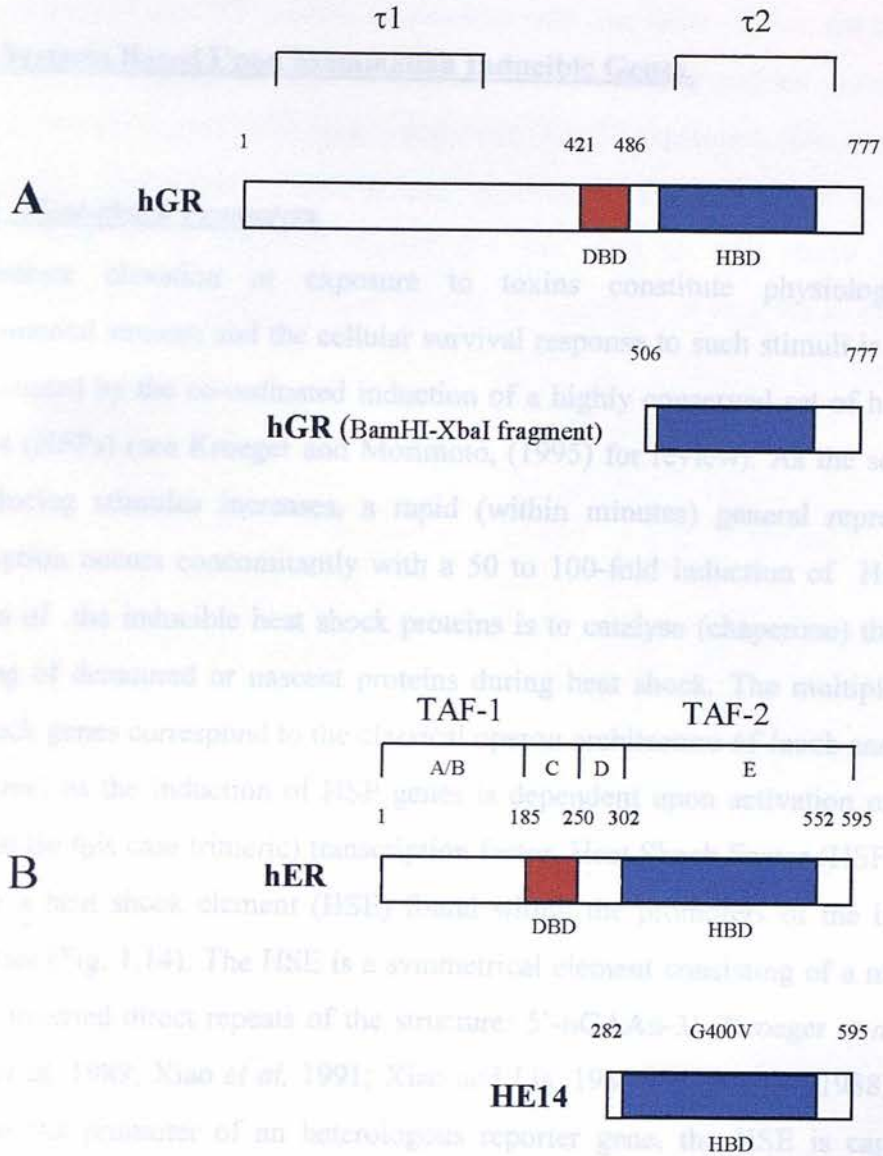


Fig. 1.13: Hormone-Binding Nuclear Receptors: GR and ER. (Data from Green and Chambon, 1991 and Eilers et al, 1987). A) Structure of the human glucocorticoid receptor (hGR) showing the DNA-binding (DBD) and hormone-binding (HBD) domains. Also indicated are the two *trans*-activating domains τ1 and τ2. The HBD used in fusions to heterologous genes is shown (lower portion). B) Structure of the hER. TAF-1 and TAF-2 are the two *trans*-activating regions. The lower portion represents the HBD (HE14) used in ER-fusion proteins.

1.2.4 Systems Based Upon Mammalian Inducible Genes.

1.2.4.1 Heat-shock Promoters.

Temperature elevation or exposure to toxins constitute physiological and environmental stresses and the cellular survival response to such stimuli is universal and mediated by the co-ordinated induction of a highly conserved set of heat shock proteins (HSPs) (see Kroeger and Morimoto, (1995) for review). As the severity of the inducing stimulus increases, a rapid (within minutes) general repression of transcription occurs concomitantly with a 50 to 100-fold induction of HSPs. The function of the inducible heat shock proteins is to catalyse (chaperone) the correct refolding of denatured or nascent proteins during heat shock. The multiple related heat shock genes correspond to the classical operon architecture of Jacob and Monod (see above) as the induction of HSP genes is dependent upon activation of a heat-regulable (in this case trimeric) transcription factor, Heat Shock Factor (HSF), which binds to a heat shock element (HSE) found within the promoters of the inducible HSP genes (Fig. 1.14). The HSE is a symmetrical element consisting of a minimum of three inverted direct repeats of the structure: 5'-nGAAn-3' (Kroeger *et al,* 1993; Perisic *et al,* 1989; Xiao *et al,* 1991; Xiao and Lis, 1988; Amin *et al,* 1988). When linked to the promoter of an heterologous reporter gene, the HSE is capable of rendering that promoter heat-inducible (Bienz and Pelham, 1986; Pelham and Bienz, 1982). The distance of the HSE from a basal promoter and co-operation between HSF and the basal promoter-binding factors determines the degree of activation of the promoter by heat (Williams and Morimoto, 1990; Greene and Kingston, 1990). Once sufficient HSPs have been produced, HSF is dissociated from the HSE owing to binding by HSP70 in an autoregulatory negative feedback loop (Abravaya *et al,* 1991; Abravaya *et al,* 1992).

Whilst promoters containing HSEs linked to heterologous genes have found a use in transgenic plants, nematodes and flies as inducible gene expression systems and as

biosensors for environmental agents, heat shock-inducible promoters have been used with heterologous reporter genes in mammalian cells, but these vectors are effectively limited to the study of inducible gene expression following cellular stress and in transgenic mice to monitor cell lineage dependent Hsp70 expression (Dix *et al*, 1996). These vectors have failed to find general applicability in experimental mammalian systems owing to the manifold stimuli that can elicit an heat shock response, significant background expression and the lack of specificity of HSF for an exogenous promoter.

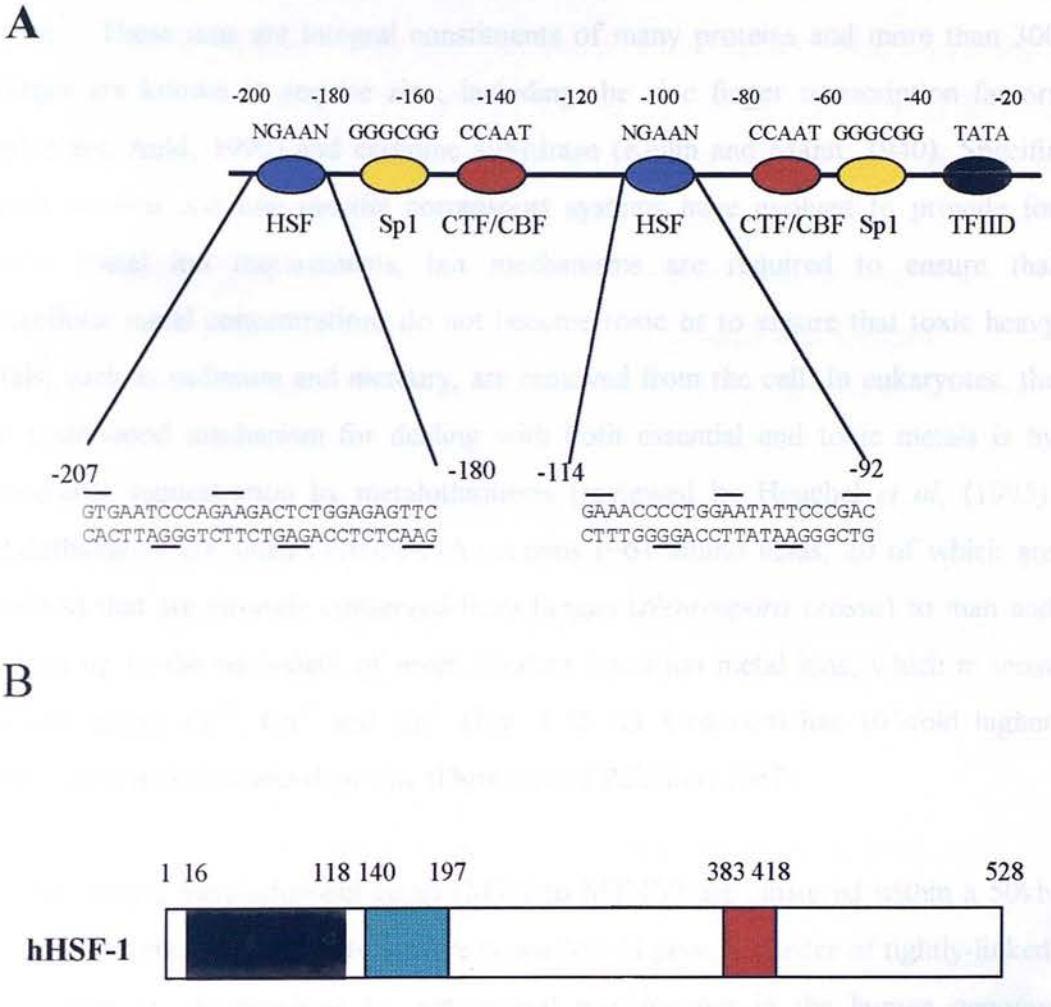


Fig. 1.14: A) Structure of the Proximal Promoter of the Human HSP70 Gene. (Kroeger and Morimoto, 1985). Indicated are the critical sequence elements of transcription factor binding sites. HSF: Heat Shock Factor; CBF: CCAAT-box-Binding Factor. CTF: CCAAT-box-Binding Transcription Factor. **B) Human Heat Shock Factor-I** (hHSF-1). Black box: DNA binding domain. Green and red boxes: Leucine zipper motifs that mediate HSF trimerisation.

1.2.4.2 Metal Responsive Promoters.

The metal ions that are essential for life as trace elements are: Zn^{2+}, Co^{2+},Ni^{2+},$Cu^{1,2+}$ and Fe^{3+}. These ions are integral constituents of many proteins and more than 300 enzymes are known to require zinc, including the zinc finger transcription factors (Vallee and Auld, 1990) and carbonic anhydrase (Keilin and Mann, 1940). Specific import systems and non-specific cotransport systems have evolved to provide for cellular metal ion requirements, but mechanisms are required to ensure that intracellular metal concentrations do not become toxic or to ensure that toxic heavy metals, such as cadmium and mercury, are removed from the cell. In eukaryotes, the best understood mechanism for dealing with both essential and toxic metals is by intracellular sequestration by metalothioneins (reviewed by Heuchel *et al*, (1995). Metalothioneins are small cysteine-rich proteins (~61 amino acids, 20 of which are cysteines) that are strongly conserved from fungus (*Neurospora crassa*) to man and can bind up to the equivalent of seven bivalent transition metal ions, which in most cells are mainly Zn^{2+}, Cu^{2+} and Cd^{2+} (Fig. 1.15 A). Cadmium has 10^4-fold higher affinity for metallothionein than zinc (Durnam and Palmiter, 1987).

The four mouse metalotheinein genes (MT-I to MT-IV) are clustered within a 50kb region on chromosome 8, whereas there is one MT-II gene, a cluster of tightly-linked MT-I genes on chromosome 16 and several pseudogenes in the human genome (Quaife *et al*, 1994; West *et al*, 1990; Searle *et al*, 1984). Regulation of metallothionein activity is at the transcriptional level, although only MT-I and -II proteins are inducible by heavy metals (Quaife *et al*, 1994; Palmiter *et al*, 1992; Durnam and Palmiter, 1981; Karin and Herschman, 1980). Multiple metal response elements (MREs: 6 in the mMT-I promoter (Fig. 1.15, B and C)) have been found within 200bp upstream of the transcriptional start site of the MT-I and -II genes and in the mMT-I gene conform to a consensus sequence: 5'-CTNTGCRCNCGGCCG-3' (the minimal core for metal responsiveness is underlined) (Mueller *et al*, 1988; Stuart *et al*, 1984). Tandemly-repeated MREs confer metal responsiveness to an heterologous reporter gene when placed upstream of a minimal promoter (TATA

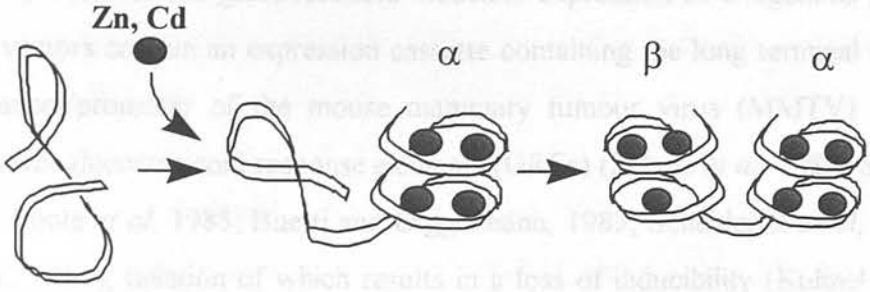
box)(Stuart *et al*, 1985) and 8 copies of the strongest MRE from the mMT-I promoter (MREd) can act as a metal-responsive enhancer when placed at a distance to an heterologous gene (Westin and Schaffner, 1988; Serfling *et al*, 1985).

The transcription factor that binds to MREs and is responsible for metal inducibility is MTF-1, a zinc-finger protein. In contrast to wild-type embryonic stem (ES) cells, MTF-1 -/- ES cells contain no detectable MT-I or MT-II mRNA either in the presence or absence of zinc, cadmium, nickel or lead. In addition, transfection of a 4xMREd reporter gene into MTF -/- stem cells produced barely detectable levels of expression with or without 400μM zinc. Restoration of basal and inducible reporter gene activity was restored to levels observed in wild-type ES cells by cotransfection of an MTF-1 expression vector (Heuchel *et al*, 1994). The requirement for MTF-1 for basal promoter activity is intriguing as it may suggest that the concentration of zinc may determine the tightness of binding of MTF-1 zinc fingers to MREs or that other factors are involved in the regulation of the transcriptional upregulation elicited by increased metal concentration (see Heuchel et al, (1995) for further discussion of these possibilities) .

metallothionein promoters from mouse, human and sheep have been used to experimentally regulate the expression of several heterologous genes, including several oncogenes (Khan *et al*, 1996; Touray *et al*, 1991; Bonham *et al*, 1991). In particular, metal responsive constructs have been successful in controlling certain circumstances where expression of an antisense RNA or a dominant-negative mutant protein has effects that inhibit cell growth and transformation (Touray *et al*, 1991; Kaplan, 1994; Schilbach *et al*, 1990). The sheep MT-Ia promoter has been particularly effective as an inducible promoter it exhibits low or negligible uninduced expression, lower than achieved by the murine MT-I promoter (Walter *et al*, 1991). Whilst it produced a graded induction of v-Myc in response to Zn2+ in Rat1 cells (Bonham *et al*, 1991), the mMT-I promoter linked to an APRT gene produced a stepped threshold response in CHO cells (Walter *et al*, 1991). Human promoters have not been so effective. The hMT-1a promoter has been observed to exert "tight, but

not absolute control of transcription" by zinc (Khan *et al*, 1996), while leaky expression from an MT-SV40 large T antigen construct in the uninduced state was sufficient to allow inappropriate cellular proliferation of primary fetal intestinal cells that resulted in no observable difference in differentiation markers between the induced and uninduced states (Paul *et al*, 1993). Further, hMT-IIa-regulated expression of an MHC-II antisense RNA transgene in the spleen was only increased 5-fold by zinc administration (Imoto *et al*, 1993). In addition, several MT promoters, including hMT-IIa, are also responsive to activation of the glucocorticoid receptor as they also contain glucocorticoid response elements (GREs)(Touray *et al*, 1991). Concerns about the responsiveness and basal activity of MT promoters, together with the known toxicity of the inducing agent (commonly cadmium or high concentrations of zinc), compromises the choice of such vectors for experiments involving the observation of the effects of apoptosis genes.

A



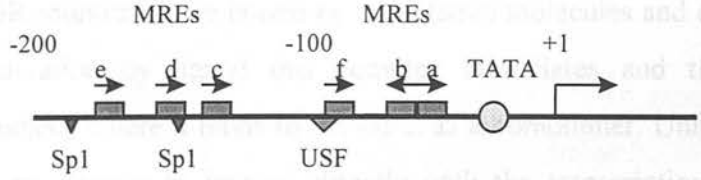
B

mMT-1

a	-54	CTTTGCGCCCGGACT	-40
b	-56	GTTTGCACCCAGCAG	-70
f	-94	CTATGCGTGGGCTGG	-80
c	-132	AAGTGCGCTCGGCTC	-118
d	-150	CTCTGCACTCCGCCC	-136
e	-175	CTGTGCACACTGGCG	-161

CONSENSUS CTNTGCRCNCGGCCG

C



mMT-I enhancer/promoter

Fig. 1.15: A) Metallothionein Binding of Metal Ions (from Heuchel *et al*,1995). **B) Metal Response Elements** from the mouse Metallothionein-I (mMT-I) gene promoter (Heuchel et al, 1995). C) **Structure of the mMT-I Promoter.** MRE: metal response element.

1.2.4.3 Glucocorticoid-Inducible Vectors.

Two mammalian expression vectors, pMAMneo (Clontech) and pMSG (Pharmacia), are commonly used for the glucocorticoid-inducible expression of exogenous genes. Both these vectors contain an expression cassette containing the long terminal repeat (LTR) enhancer/promoter of the mouse mammary tumour virus (MMTV) which contains several glucocorticoid response elements (GREs) (Schule *et al*, 1988; Strahle *et al*, 1988; Ponta *et al*, 1985; Buetti and Diggelmann, 1983; Scheidereit *et al*, 1983; Payvar *et al*, 1983), deletion of which results in a loss of inducibility (Kuhnel *et al*, 1986; Hynes *et al*, 1983). The response to glucocorticoids (such as dexamethasone (DEX)) and progestins is mediated by binding of the appropriate ligand-bound hormone receptor to the GREs. Whilst the progesterone receptor (PR) has a more limited cell-type distribution, the glucocorticoid receptor (GR) is expressed in nearly all mammalian cells. The GR was the first identified and characterised member of the steroid receptor superfamily (Evans, 1988) and like the other members (see above) has three main domains: an N-terminal domain which contains the τ1 transcriptional activation activity; a DNA binding domain which specifically recognises the GRE sequence by two zinc finger motifs; and a C-terminal hormone binding domain which, in addition to mediating ligand binding, also contains the τ2 *trans*-activation function as well as the determinants for specific nuclear localisation and dimerisation (reviewed in Eggert *et al*, (1995)). In the non-ligand bound state, the inactive GR is located in the cytoplasm and GR monomers are bound by two Hsp90 molecules and one Hsp59 molecule. Upon activation by ligand this complex dissociates and the GR is transported to the nucleus where it binds to the GRE as a homodimer. Unlike the PR and the ER, which are known to interact directly with the transcription initiation complex (TIC) by contacts with TFIIB (Ing *et al*, 1992), such an interaction has not been proven for the GR. However, the GR is known to synergise with other transcription factors (e.g. AP-1) bound to adjacent sites on glucocorticoid-inducible promoters, possibly by co-operative binding or via other accessory factors (Eggert *et al*, 1995).

In rat hepatoma cells, expression from integrated MMTV proviral DNA is suppressed by a putative repressor protein and the maximal inducibility of viral mRNA in these cells is determined by the relative level of GR (Tanaka *et al*, 1993). Accordingly, synthetic promoters containing multiple GREs are highly inducible in cells that overexpress the GR (Israel and Kaufman, 1989) or PR (Mader and White, 1993). Cells transfected with MMTV-based vectors containing both the GR and reporter genes result in positive feedback when treated with DEX and this results in high inducibility factors, in one case of the order of 10^4-fold without a significant increase in background expression (Ko *et al*, 1989). The glucocorticoid-inducible system has found use with a wide variety of genes, including the dexamethasone-regulated production of modest amounts of wild-type p53 in a glioblastoma cell line (Mercer *et al*, 1990). This cell line was exploited by El-Deiry *et al*, (1993) to clone the p53 responsive-gene $p21^{WAF1}$ by subtractive hybridisation. However, like the heat shock- and interferon-inducible systems, glucocorticoid-inducible vectors suffer from the pleiotropic nature of the cellular response to the inducing agent and therefore requires careful consideration of control experiments. More importantly, dexamethasone has been shown to induce apoptosis of thymocytes (Wyllie *et al*, 1980), thus limiting the general applicability of this system for the study of apoptosis genes in transgenic animals.

1.2.4.4 Other Inducible Mammalian Promoters.

Other promoters that have been used for the regulation of reporter genes either in transfected cell lines and in transgenic mice include several induced by interferon or double stranded RNA (p(I:C)) (including that from the human 6-16 gene) (Chernajovsky and KirbySanders, 1990), retinoic acid (Colbert *et al*, 1993) and dioxin (De Benedetti and Rhoads, 1991). All of these systems suffer from similar fundamental drawbacks (leakiness and pleiotropism and (or) toxicity of inducer) to the other mammalian promoter systems described above when considered for use with apoptosis or cell cycle regulatory genes.

1.2.5 Inducible Promoters Containing Bacterial or Yeast Gene Control Elements.

The probability of a specific 18bp nucleotide sequence occurring at random is $(0.25)^{18}$ which corresponds to an incidence of 0.04 times per haploid mammalian genome of $3x10^9$bp. Any similar sequence differing by no more than four substitutions is likely to occur at random with a probability of less than $4x10^{-9}$ (13 times in the mouse genome). Since 1% of the genome contains genes it is likely that sequences similar to prokaryotic and yeast DNA binding protein recognition sites will occur in mammalian genes at a frequency of less than 0.13 per gene (Liu *et al*, 1992) and at lower frequencies at sites critical for gene regulation. Therefore, the interaction of prokaryotic or yeast DNA binding proteins to their cognate recognition sites is likely to be highly specific to exogenous constructs in mammalian cells. This is the basis of the use of such genetic elements in experimental inducible gene systems.

1.2.5.1 Mammalian Inducible Expression Systems based upon the E.coli lac Operon.

The *E. coli lac* operon was the first gene regulation system to be described and characterised and provided confirmation of the predictions of Jacob and Monod concerning the organisation of gene structure and function (Jacob and Monod, 1961). The response of E. coli to the presence of the sugar lactose in its environment it to induce the expression of three enzyme activities from a single genomic polycistronic transcriptional unit up to 1000-fold: β-galactosidase (β-D-galactoside galactohydrolase E.C.3.2.1.23), the product of the *lac*Z gene that is responsible for hydrolysis of galactoside substrates to galactose; lactose permease (*lac*Y); and thiogalactoside acetylase (*lac*A)(Fig. 1.16).

The *E. coli lac* Operon

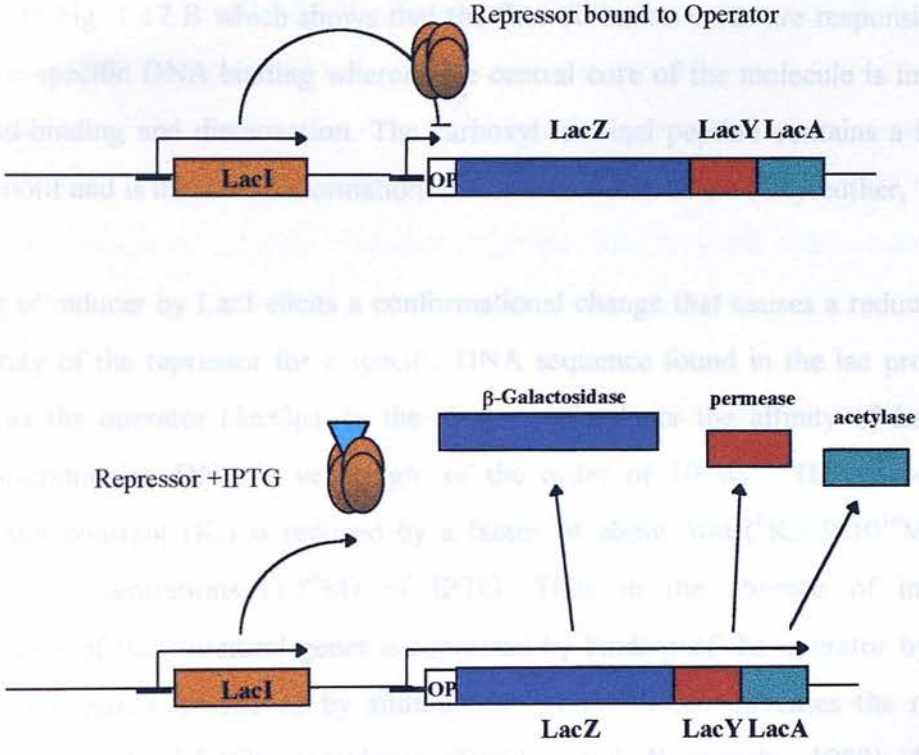


Fig. 1.16: The *E. coli lac* Operon. OP: lac operator sequence.

Allolactose is both the natural inducer and substrate of the *lac* operon and is produced from lactose by β-galactosidase itself by the transfer of the galactosyl group from the 4 to the 6 position of glucose (Zabin and Fowler, 1980). Allolactose and the synthetic non-hydrolysable inducer IPTG (isopropyl-β-D-thiogalactoside) bind to a homotetrameric repressor protein (the product of a separate, but closely-linked, constitutively expressed gene: *lac*I). The functional domains of the LacI protein monomer have been determined by extensive mutational and physical analysis and are outlined in Fig. 1.17 B which shows that the first 60 amino acids are responsible for sequence-specific DNA binding whereas the central core of the molecule is involved in ligand-binding and dimerisation. The carboxyl-terminal peptide contains a leucine zipper motif and is involved in formation of stable homotetramers (Beyreuther, 1980).

Binding of inducer by LacI elicits a conformational change that causes a reduction in the affinity of the repressor for a specific DNA sequence found in the lac promoter known as the operator (*lac*Op). In the absence of inducer the affinity of LacI for operator-containing DNA is very high: of the order of $10^{13}M^{-1}$. This association equilibrium constant (K_o) is reduced by a factor of about 300 ($^{E}K_o=3x10^{10}M^{-1}$) by saturating concentrations ($10^{-4}M$) of IPTG. Thus in the absence of inducer, transcription of the structural genes is repressed by binding of the operator by LacI and this repression is relieved by addition of inducer which increases the rate of dissociation of LacI/*lac*Op complexes (Barkley and Bourgeois, 1980). DNase protection and filter-binding studies have indicated that the binding of the repressor to the operator (-3 to +21) and binding of RNA polymerase (-24 to +19) to the *lac* promoter is mutually-exclusive (Reznikoff and Abelson, 1980; Gilbert, 1976; Gilbert and Maxam, 1973). The nucleotide sequence of the natural operator revealed a high degree of symmetry but also showed that the sequence was not perfectly palindromic (Fig. 1.17 A). DNase protection studies using a battery of chemically modified or substituted *lac*Op sequences showed that alterations to residues making critical contacts with LacI in the left half of the operator had a much greater affect on repressor binding than did corresponding alterations at symmetrical sites in the right half. For example, the thymidine methyl group at position 8 of the wild-type *lac*Op

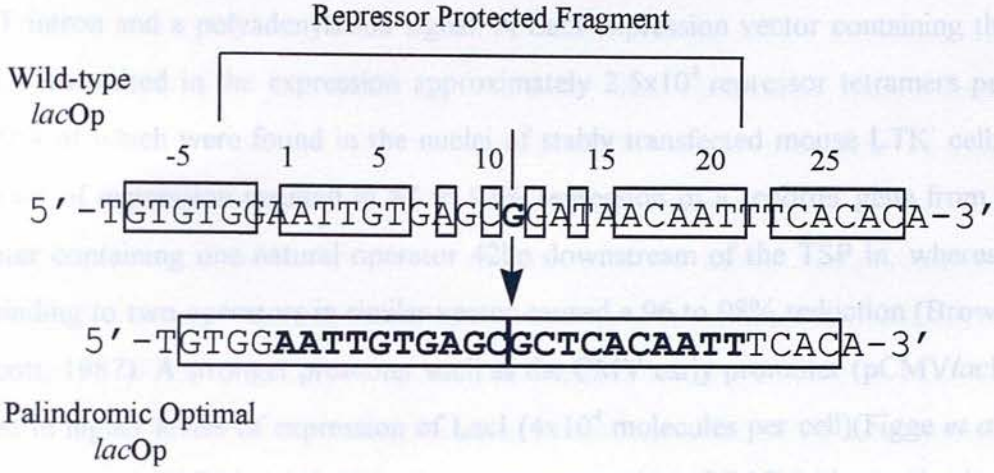
mediates a 12-fold increase in affinity for LacI when compared to a mutant operator site containing a uracil substitution at that position. The corresponding substitution in the right half of the operator at position 14 does not significantly affect the affinity of repressor binding. Experiments with synthetic, perfectly symmetrical operator fragments based upon the sequence of the left half and lacking the central G-C pair showed that repressor bound with 8 to 11-fold higher affinity *in vitro* whilst still retaining the ability to release the operator in the presence of IPTG. It has been suggested that the palindromic 'ideal' operator site is bound symmetrically by a LacI tetramer by two (or two pairs of) subunits whereas the wild-type sequence is only bound strongly by one (or one pair) subunit asymmetrically. A symmetrical oligonucleotide composed of the right half sequence (Fig. 1.17 A) binds LacI only 5 to 10% as strongly as does the wild-type sequence (Simons *et al,* 1984; Sadler *et al,* 1983).

The lac repressor protein has been shown to bind to operator sites in DNA that has been packaged into nucleosomes *in vitro* (Chao *et al,* 1980) and has been shown inhibit transcription from several eukaryotic and viral promoters (SV40 early promoter, RSV-LTR, mMT-I and a semi-synthetic MSV-SV40 early promoter) containing operator sites at various positions relative to the TATA box and endogenous transcription start points (TSPs)(Figge *et al,* 1988; Brown and Scott, 1987; Hu and Davidson, 1987)(see below). Thus chromatin structure does not inhibit LacI protein expressed in mammalian cells from specifically interacting with *lac*Op DNA. Indeed, the strong non-specific DNA binding activity of LacI for naked prokaryotic DNA is diminished by the presence of histones (Chao *et al,* 1980). The inducer of the *lac* system, IPTG is taken up into mammalian cells rapidly, exceeding extracellular levels within 2 hours and the concentration in the nucleus slightly exceeds that of the cytoplasm. IPTG is cleared equally rapidly upon withdrawal from the medium. In mice, IPTG is cleared from the blood with a half-life of 15-30 minutes. In addition, IPTG has apparently no toxic or other side effects at concentrations up to 50mM (Wyborski and Short, 1991). Owing to these observations, the *lac* repressor has been developed as an inducible modulator of mammalian transcription.

Fig. 1.17: A) Natural and Synthetic Ideal lac Operators. (Adapted from Barkley and Bourgeois, 1980 and Sadler *et al.* 1983) Symmetric portions of the sequence are boxed. The axis of symmetry is indicated with an arrow and the central nucleotide position is indicated in the natural operator by bold type. In the ideal operator, the minimal sequence used in experimental inducible systems is in bold type. **B) Modifications of the *lac* Repressor for use in Mammalian Cells.** (Data from Fieck *et al.* 1992). GTG: prokaryotic translation initiation codon. TGA: stop codon. Kozak ATG: consensus eukaryotic translation initiation codon. DBD: DNA-binding domain. NLS: nuclear localisation signal

A

Lac **Operators**

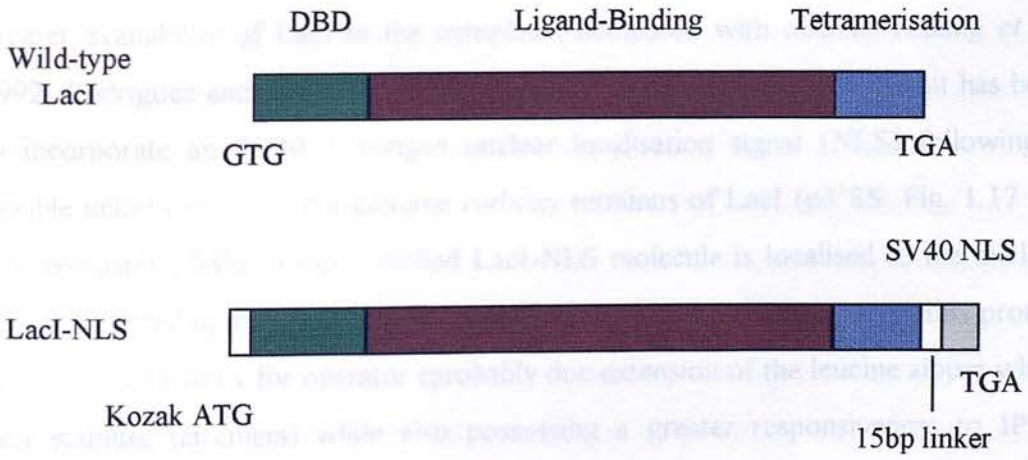


Barkley And Bourgeois, 1980; Sadler et al, 1983.

B

Modifications of the *lac* repressor for use in mammalian cells

Fieck et al, 1992



Several plasmid vectors have been constructed that direct the expression of LacI in mammalian cells. These consist of standard constitutive mammalian expression cassettes containing an enhancer/promoter element; the *lac*I gene with modifications such as the conversion of the E. coli GTG prokaryotic translation initiation codon by a eukaryotic ATG sequence; and RNA processing signals: most commonly, the SV40 small T intron and a polyadenylation signal. A LacI expression vector containing the RSV-LTR resulted in the expression approximately $2.5x10^4$ repressor tetramers per cell, 10% of which were found in the nuclei of stably transfected mouse LTK^- cells. This level of expression resulted in 87 to 92% repression of a reporter gene from a promoter containing one natural operator 42bp downstream of the TSP in, whereas LacI binding to two operators in similar vector caused a 96 to 98% reduction (Brown and Scott, 1987). A stronger promoter such as the CMV early promoter (pCMV*lac*I) resulted in higher levels of expression of LacI ($4x10^4$ molecules per cell)(Figge *et al*, 1988) and greater inhibition (98.5%) of transient expression of CAT (chloramphenicol acetyl transferase: an E. coli gene commonly used as a reporter) from an SV40 early promoter containing a single optimal *lac*Op sequence overlapping the TSP (on pSV*lac*OCAT) than a similar LacI expression construct (pMT*lac*I) containing the weaker mMT-I promoter (Brown and Scott, 1987). The cytoplasmic virus, vaccinia (VV) has been modified by several groups to express LacI. Repressor-mediated inhibition of expression of virus-encoded proteins whose promoter regions contain operator sites has been reported to have be greater than 99.9%. This may be due to greater availability of LacI in the cytoplasm compared with nucleus (Zhang *et al*, 1992; Rodriguez and Smith, 1990; Fuerst *et al*, 1989). A further refinement has been to incorporate an SV40 T antigen nuclear localisation signal (NLS) following a flexible linker peptide at the extreme carboxy terminus of LacI (p3'SS: Fig. 1.17 B). Approximately, 98% of this modified LacI-NLS molecule is localised to the nucleus thereby increasing the effective nuclear repressor concentration. *In vitro*, this protein has increased affinity for operator (probably due extension of the leucine zipper which may stabilise tetramers) while also possessing a greater responsiveness to IPTG (Fieck *et al*, 1992).

In order to confer repression by LacI to a test gene, it is necessary to insert one or more *lac*Op sites into the promoter region. Initial experiments by Hu and Davidson (1987), investigated the optimal number and position of 40bp wild-type operator sites in an SV40-derived promoter linked to a Moloney sarcoma virus (MSV) enhancer (Fig. 1.18 A). Insertion of these sequences decreased the expression of a reporter gene in the absence of LacI in all positions tested and the effect increased with increasing copy number. The inhibition was least when operator(s) were inserted between the TATA box and the endogenous TSP where, with one operator (pSMB01CAT), expression of the CAT reporter gene reached 80% of the level produced by the parental vector. Three tandem operators caused almost complete inhibition. Inhibition was greatest when operators were placed upstream of the TATA box (thereby weakening binding of transcription factors to the transcription initiation complex) or downstream of the TSP (probably by causing inhibition of translation by forming hairpin secondary structures in the 5' UTR of the CAT mRNA: this is avoided in the Lacswitch vectors pOPRSVICAT and pOPI3CAT by placement of operators downstream of the TSP in an intron (DuCoeur *et al*, 1992)). In the presence of LacI, expression was repressed (see above) and was induced to a modest degree in 20mM IPTG. However, induced expression was never fully returned to unrepressed levels.

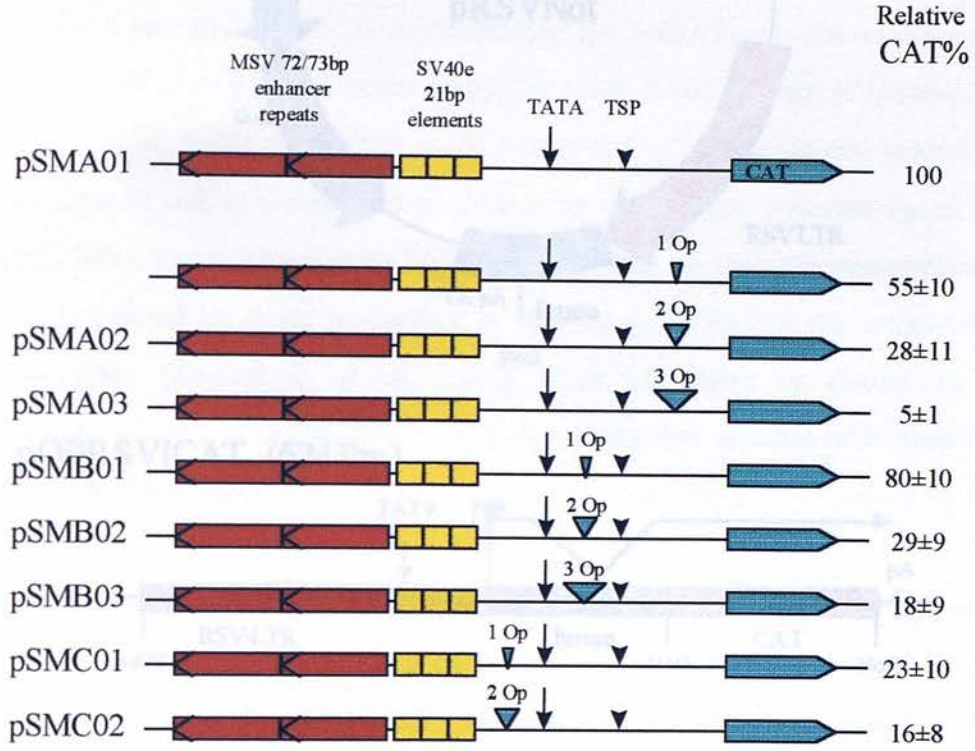
Brown *et al*, (1987) constructed a reporter vector (pSV*lac*OCAT) containing a single 18bp optimal operator site inserted as a 22bp linker overlapping the TSP of the SV40 early promoter (Fig. 1.18 B). This insertion had no effect upon the maximal levels of expression from this promoter in the absence of LacI when compared with the parental vector. In transient co-transfections of pSV*lac*OCAT and pCMV*lac*I, IPTG relieved repression by ~60%. However, the relative difference between induced and uninduced CAT expression decreased as the concentration of pSV*lac*OCAT plasmid increased (i.e. titration of the available LacI molecules with operator DNA) until the operator-containing construct became saturating. Therefore, definitive induction experiments were carried out in doubly stable transfectants (Figge *et al*, 1988). In these stable transfectants with single copy integration of the operator construct, an

induction ratio of 60 was measured following exposure to 50mM IPTG. A plateau in the induction response was not reached as IPTG concentrations in excess of 50mM were toxic. Sufficient LacI was present to mediate >99.9% repression and uninduced expression was not significantly higher than background. These observations may indicate that other, similar constructs with stronger promoters, such as the CMV early promoter, combined with more responsive LacI molecules (such as the LacI-NLS that is produced from p3'SS) may allow larger induction ratios.

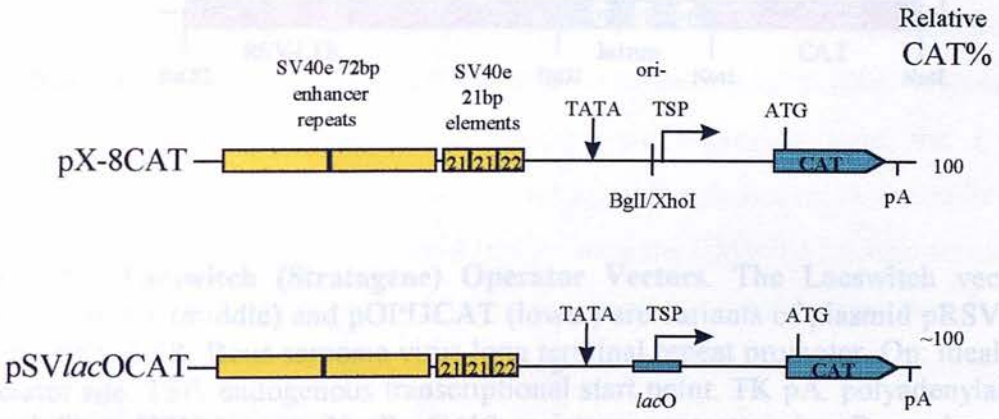
The optimised IPTG-inducible vector system (Lacswitch: Statagene) used in this project consisted of the LacI-NLS expression vector p3'SS (Fieck *et al*, (1992) and see Chapter 2: General Materials and Methods) and two alternative lac operator-containing reporter vectors: pOPRSVICAT and pOPI3CAT (Fig. 1.19). pOPRSVICAT contains two optimal operators: one between the TATA box and the endogenous TSP of the RSV-LTR; and one in an intron downstream of the TSP. pOPI3CAT contains 3 intronic optimal operator sites. Previously, the largest induction ratio with 5mM IPTG (10-fold lower than toxic doses) obtained in an individual clone derived from a stable cotransfection with p3'SS and pOPRSVILuc (a related plasmid to pOPRSVICAT) was 48-fold higher than the basal repressed expression level that was equivalent to 10-20 luciferase molecules per cell. 95-fold induction from a slightly lower basal level was observed in a clone derived with pOPI3Luc. Induction of luciferase was rapid: maximal between 4-12 hours (DuCoeur *et al*, 1992). The properties of these vectors, combined with the favourable pharmacokinetics, low toxicity and specificity of IPTG made this system an attractive choice for the purposes of this project.

Fig. 1.18 (overleaf)**: A) Effect of Copy Number and Position of Insertion of 40bp Natural Operator sites into a promoter**.(data from: Hu and Davidson, 1987). The effect of operator insertion on promoter-driven CAT (chloramphenicol acetyl transferase) activity in the absence repressor protein. MSV: Moloney murine sarcoma virus. SV40e: SV40 early promoter. TSP: transcriptional starting point. Op: 40bp natural operator sequence. **B) Insertion of an ideal *lac*Op site at the TSP** (Data from Brown *et al*, 1987; Figge *et al*, 1988). The insertion of a single ideal lac operator site (*lac*O) overlapping the TSP has no significant effect on promoter-driven CAT activity. Ori-: a deletion of the SV40 origin of replication in the parental vector (pX-8CAT). pA: polyadenylation signal

A



B Insertion of an ideal *lac*Op site at the TSP (Brown *et al*, 1987; Figge *et al*, 1988)



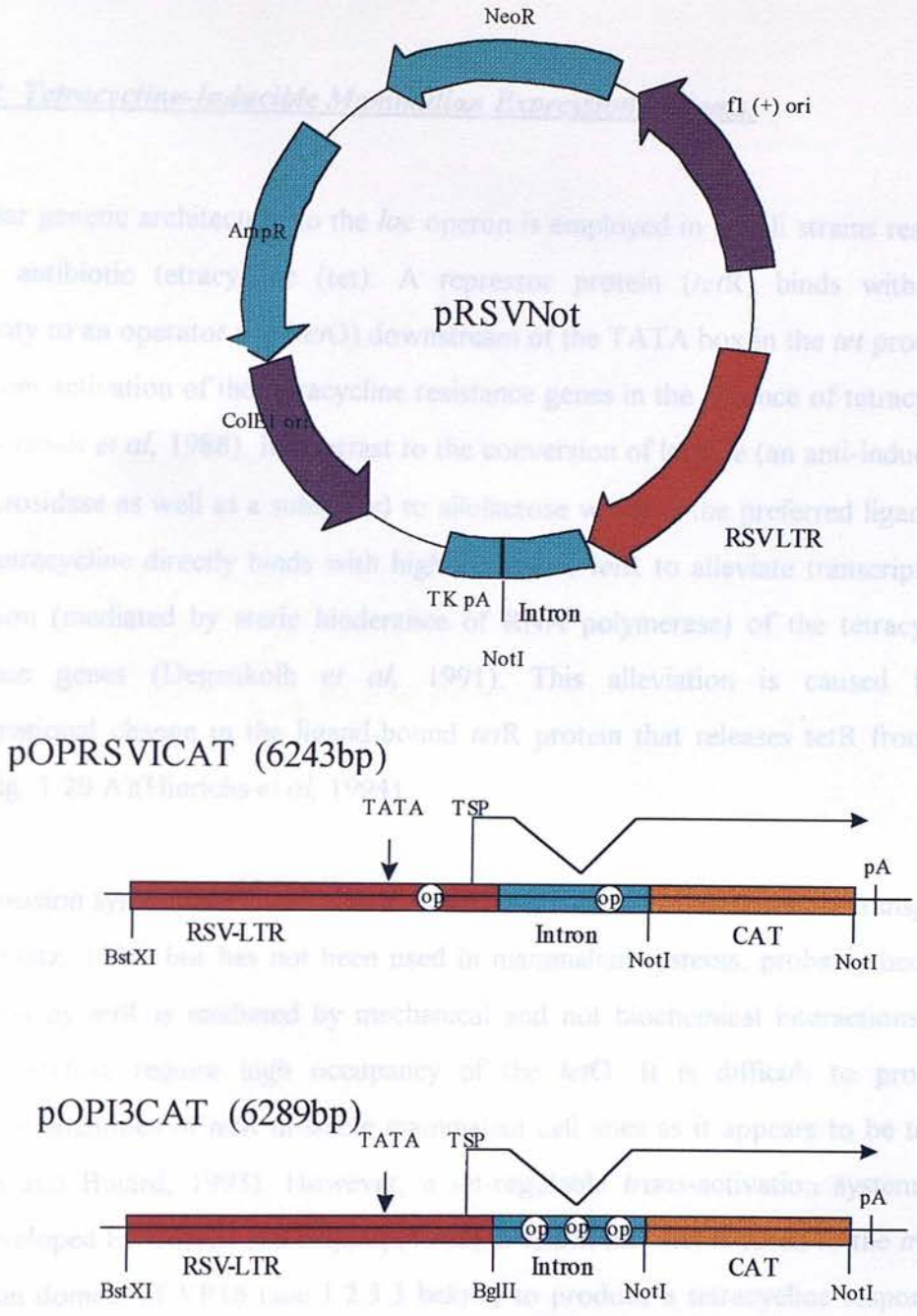


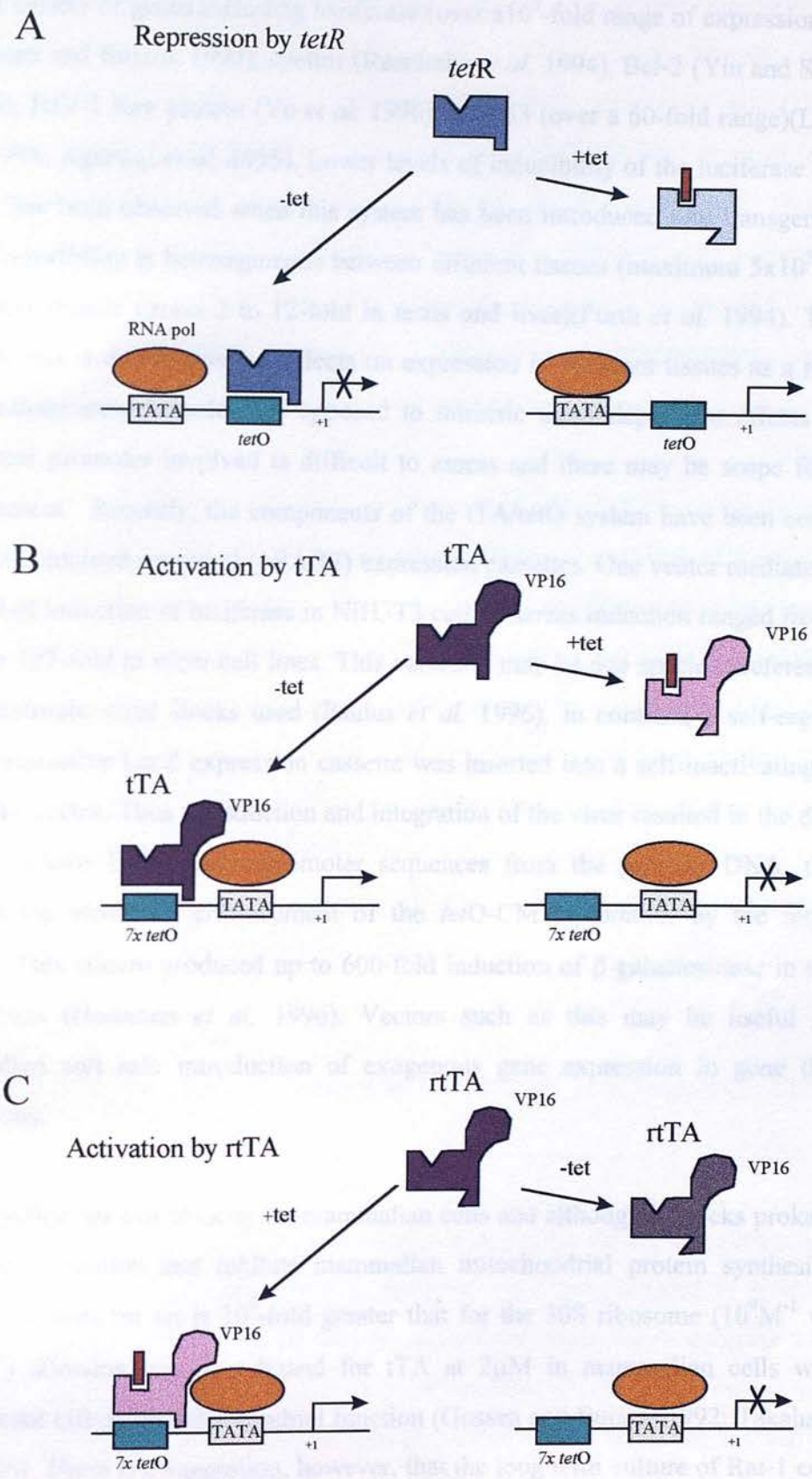
Fig. 1.19: Lacswitch (Stratagene) Operator Vectors. The Lacswitch vectors pOPRSVICAT (middle) and pOPI3CAT (lower) are variants of plasmid pRSVNot (Top). RSV-LTR: Rous sarcoma virus long terminal repeat promoter. Op: ideal lac operator site. TSP: endogenous transcriptional start point. TK pA: polyadenylation signal from HSV*tk* gene. NeoR: G418 resistance cassette. AmpR: prokaryotic ampicillin resistance (β-lactamase) gene. Ori: prokaryotic (ColE1) or filamentous phage (f1 (+)) origin of replication. CAT: chloramphenicol acetyl transferase gene.

1.2.5.2 Tetracycline-Inducible Mammalian Expression Systems.

A similar genetic architecture to the *lac* operon is employed in E.coli strains resistant to the antibiotic tetracycline (tet): A repressor protein (*tet*R) binds with high specificity to an operator site (*tet*O) downstream of the TATA box in the *tet* promoter to prevent activation of the tetracycline resistance genes in the absence of tetracycline (Kleinschmidt *et al,* 1988). In contrast to the conversion of lactose (an anti-inducer of β-galactosidase as well as a substrate) to allolactose which is the preferred ligand for LacI, tetracycline directly binds with high affinity to *tet*R to alleviate transcriptional repression (mediated by steric hinderance of RNA polymerase) of the tetracycline resistance genes (Degenkolb *et al,* 1991). This alleviation is caused by a conformational change in the ligand-bound *tet*R protein that releases tetR from the *tet*O (Fig. 1.20 A)(Hinrichs *et al,* 1994).

An expression system based upon the *tet*R/*tet*O interaction has been used in transgenic plants (Gatz, 1996) but has not been used in mammalian systems, probably because repression by *tet*R is mediated by mechanical and not biochemical interactions and would therefore require high occupancy of the *tet*O. It is difficult to produce detectable quantities of *tet*R in stable mammalian cell lines as it appears to be toxic. (Gossen and Bujard, 1993). However, a tet-regulable *trans*-activation system has been developed by Gossen and Bujard, (1992) in which the *tet*R is fused to the *trans*-activation domain of VP16 (see 1.2.5.3 below) to produce a tetracycline responsive *trans*-activator (tTA) protein. When constitutively expressed from the CMV immediate early promoter, the tTA binds to and strongly activates transcription from a minimal promoter on a separate plasmid that contains the CMV TATA box devoid of enhancers via seven tandem copies of the *tet*O placed immediately upstream. In the presence of tet, the tTA is released from the promoter and the transcription rate drops to a low level (Fig. 1.20 B).

Fig. 1.20: A) Repression by *tetR*. (From Shockett, 1996) In the absence of tetracycline (tet), the *tet*R binds to the *tet*O and thereby represses transcription from an upstream promoter. When tetracycline is present and binds to the repressor transcription occurs as a result of release of the *tet*R from the *tet*O. RNA pol: RNA polymerase **B) Conversion of the *tet*R to a transcriptional activator** by fusion with the transcriptional activation domain of VP16 (tTA). **C) Reverse tetracycline *trans*-activator** (rtTA).


A
Repression by tetR
tetR
+tet
-tet
RNA pol
TATA
tetO
+1
TATA
tetO
+1
B
Activation by tTA
tTA
VP16
+tet
VP16
-tet
tTA
VP16
TATA
7x tetO
+1
TATA
7x tetO
+1
C
rtTA
VP16
Activation by rtTA
rtTA
-tet
VP16
+tet
VP16
TATA
7x tetO
+1
TATA
7x tetO
+1

This basic system has been extensively used in tissue culture for the expression of wide variety of genes including luciferase (over a 10^5-fold range of expression levels) (Gossen and Bujard, 1992), cyclins (Resnitzky *et al,* 1994), Bcl-2 (Yin and Schimke, 1996), HIV-1 Rev protein (Yu *et al,* 1996) and p53 (over a 60-fold range)(Lassus *et al,* 1996; Agarwal *et al,* 1995). Lower levels of inducibility of the luciferase reporter gene has been observed when this system has been introduced into transgenic mice and inducibility is heterogeneous between different tissues (maximum $5x10^3$-fold in seminal vesicle versus 2 to 12-fold in testis and liver)(Furth *et al,* 1994). To what extent this is due to position effects on expression in different tissues as a result of the transgenesis procedure as opposed to intrinsic tissue-dependent effects on the minimal promoter involved is difficult to assess and there may be scope for more refinement. Recently, the components of the tTA/tetO system have been combined in self-contained retroviral (pBABE) expression cassettes. One vector mediated up to 336-fold induction of luciferase in NIH3T3 cells whereas induction ranged from only 24- to 127-fold in other cell lines. This variation may be due species preferences by the ecotropic virus stocks used (Paulus *et al,* 1996). In contrast, a self-regulatory tTA-responsive LacZ expression cassette was inserted into a self-inactivating (SIN) pBABE vector. Thus transduction and integration of the virus resulted in the deletion of the vector U3 enhancer/promoter sequences from the proviral DNA, thereby preventing unwanted enhancement of the *tet*O-CMV promoter by the retroviral LTRs. This system produced up to 600-fold induction of β-galactosidase in murine myoblasts (Hofmann *et al,* 1996). Vectors such as this may be useful in the controlled and safe introduction of exogenous gene expression in gene therapy situations.

Tetracycline has low toxicity for mammalian cells and although it blocks prokaryotic peptide elongation and inhibits mammalian mitochondrial protein synthesis, the affinity of *tet*R for tet is 10^3-fold greater that for the 30S ribosome (10^9M^{-1} versus 10^6M^{-1}) allowing use as a ligand for tTA at 2μM in mammalian cells without significant effects on mitochondrial function (Gossen and Bujard, 1992; Takahashi *et al,* 1986). There is a suggestion, however, that the long term culture of Rat-1 cells in

toxicity (Dr. C. Boyle, personal communication). It is not known whether tet analogues such as anhydrotetracycline may circumvent this as they bind to tetR with much greater affinity ($10^{11}M^{-1}$: (Hecht *et al,* 1993)) enabling the use of lower concentrations of ligand. The response of the tTA system to withdrawal of tetracycline from cell cultures is slow. It takes up to 48 hours for a maximal response to occur as tetracycline is eliminated from cells only by passive diffusion. This means that this system is unsuitable for the study of short term effects of gene expression. As apoptosis is often completed within 24 hours following a stimulus, the tTA system is not ideal for the expression of apoptosis inducing genes, despite the attraction of the low basal and high induced expression levels achievable.

In an important recent development, a mutant tetR has been isolated that has an inverted response to ligand enabling the construction of a reverse tTA (rtTA) that binds to tetO only in the presence of tet analogues doxycycline or anhydrotetracycline (Fig. 1.20 C)(Gossen *et al,* 1995). Using this modified system luciferase activity was induced 10^3-fold in 20 hours in a dose-dependent manner. It has been proposed that this latest refinement of the tet system could be useful in situations where long term exposure to tetracycline is undesirable or inconvenient (e.g. in vivo gene therapy or transgenic animals) or where the slow (up to 48hours) passive elimination of tet from cells in culture or animals may confound studies where rapid induction is required (Shockett and Schatz, 1996; Gossen and Bujard, 1992).

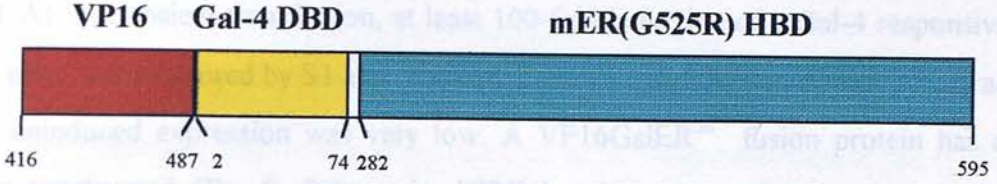
1.2.5.3 A Synthetic Tamoxifen-Inducible Mammalian Gal4-Responsive Promoter System.

The occurrence of separable functional domains in a wide variety of protein families has been suggested to reflect that in evolution (and recapitulated in lymphocyte ontogeny), elements from a small pool of genes were combined to generate a larger pool of genes containing a diverse selection of activities. Transcription factors contain at least two functional domains: a domain that mediates sequence-specific DNA binding and one that has effects on transcriptional activation. As discussed in the

section on fusion proteins above, the nuclear hormone receptor family also contain ligand-binding regulatory domains that may overlap with *trans*-activation activities. In a similar manner to evolutionary processes, the molecular biologist can dissect genes and recombine the fragments *in vitro* to generate proteins with novel functions. Such technology has been exploited in the generation of novel transcription factor activity whose function is ligand-dependent: VP16GalER (Braselmann *et al*, 1993).

The first 74 amino acids of the *Saccharomyces* Gal-4 transcription factor contain a zinc-finger DNA binding domain (Sollerbrant *et al*, 1995) that binds specifically to the 17bp sequence: 5'-CGGAGGACAGTCCTCCG-3'. The transcriptional activation domain of intact Gal-4 thus more readily able to exert its effects at a promoter containing a Gal-4-binding site than in free solution. This enhancement of transcription by Gal-4 does not require both activities on the same polypeptide but so long as there is a physical association between two proteins each contain one of the separated domains of Gal-4, then specific transctivation of a reporter gene can occur. This is the basis of the 2-hybrid interaction screen which is a genetic assay for interacting proteins *in vivo*. Fusion of the Gal-4 DBD to an heterologous transcriptional activity can also result in novel transcriptional activity. One such fusion protein (Gal4-VP16) involves the C-terminal trans-activation domain (amino acids 416-487) from the *Herpes simplex* virus (HSV) VP16 (Vwm65) protein (Triezenberg *et al*, 1988a). VP16 is a component of the HSV virion and mediates the immediate early transcriptional response by binding via N-terminal sequences to host proteins bound to promoters thus bringing the highly acidic trans-activation domain into contact with the transcriptional machinery (McKnight *et al*, 1987; Preston *et al*, 1988; O'Hare and Goding, 1988; Triezenberg *et al*, 1988; Triezenberg *et al*, 1988). Gal4-VP16 is unusually efficient at stimulating transcription when bound to Gal-4 recognition sites placed close to, or at a distance (at least 1.8kb) from a mammalian promoter (Sadowski *et al*, 1988).

A



B

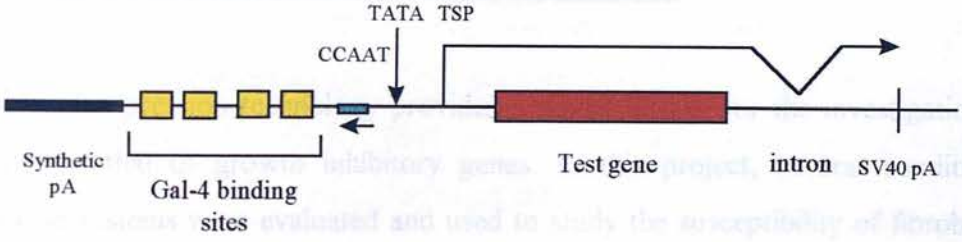


Fig. 1.21: A) VP16GalER^{tm} Fusion Protein. (constructed by Dr. S. Pellengaris). VP16: *Trans*-activation domain from Herpes simplex virus VP16 protein. Gal-4 DBD: Gal-4 DNA binding domain. mER(G525R) HBD: mutant mouse (tamoxifen-sensitive) oestrogen receptor hormone binding domain. **B) Structure of a synthetic Gal-4-responsive mammalian promoter** (Braselmann *et al*, 1993). pA: polyadenylation signal. TSP: transcription start point.

A tripartite fusion protein, Gal-ER-VP16 and a synthetic Gal-4 responsive mammalian promoter (see Chapter 2: Materials and Methods and Fig. 1.21 B) was constructed by Braselmann et al, (1993). The chimaeric protein takes advantage of the strong transcriptional activity of the VP16 trans-activation domain, the exogenous sequence specificity of Gal-4 combined with regulation by the oestrogen receptor HBD (Fig. 1.21 A). In transient transfection, at least 100-fold induction of a Gal-4 responsive c-Fos gene was measured by S1 analysis within 1-2 hours of the addition of β-oestradiol and uninduced expression was very low. A VP16GalER^{tm} fusion protein has also been constructed (Dr. S. Pelengaris, ICRF London: personal communication)(see Chapter 2: Materials and Methods for details) that contains the mouse ER(G525R) mutant that is responsive only to tamoxifen. This construct was assessed in this project.

1.2.6 Conclusion: Conditional Apoptosis Experiments.

Conditional expression technology provides a powerful tool for the investigation of potentially lethal or growth inhibitory genes. In this project, several conditional expression systems were evaluated and used to study the susceptibility of fibroblasts to undergo apoptosis under conditions where the expression of individual test genes could be exogenously-regulated. Using the temperature-sensitive p53, the susceptibility of fibroblasts to chemotherapeutic drug treatment was examined in the case of overexpression of phenotypically wild-type or mutant p53. Experiments were designed to show that the outcome of activation of wild-type p53 (by using either the temperature-sensitive mutant or by controlling levels of p53 expression by using the Lacswitch or VP16GalER^{tm} systems) could be altered by changes in the cellular environment such as co-expression of c-myc (using the *myc*-ER fusion protein and (or) the Lacswitch or VP16GalER^{tm} systems). Inducible p21$^{WAF1/CIP1}$ expression vectors were constructed with the intention of producing cell lines which undergo an IPTG- or tamoxifen-dependent cell cycle arrest but grew normally in the absence of

inducer. Such constructs could be used, together with appropriate c-*myc* expression constructs, to test whether p21 could substitute for p53 in triggering *myc*-driven apoptosis. It is known that several treatments, such as thymidine block or isoleucine starvation which elicit cell cycle arrest even when cells are cultured with serum is sufficient to cause Rat1/*myc* cells to undergo apoptosis (Evan *et al*, 1992). It is important to identify the function of p53 that is responsible for the induction of apoptosis as this could have an impact upon the direction of anticancer research, especially for the targets of gene-based therapeutic strategies.

A rapidly expanding area of apoptosis research is the characterisation of the ICE-like proteases. Much effort has been focused upon the structure and mechanism of action of these enzymes. Identification of physiological substrates has been hampered by observations that individual proteases can activate other family members. Assays of the biological activity of a newly identified protease in live cells often include a transient overexpression experiment. These assays are, for several reasons, not conclusive proof that a particular protease forms part of the apoptosis effector pathway. The most common criticism of this approach is that loading of cells with any protease may be sufficient to cause the death of cells by a process that resembles apoptosis (Williams and Henkart, 1994). The advent of highly-regulable and titratable inducible-expression systems may circumvent this problem.

Chapter 2.

2. General Materials and Methods

2.1 Eukaryotic Expression Vectors

2.1.1 pBKCMV and pPUR

pBKCMV (Stratagene) (Fig. 2.1) is an expression/cloning vector that contains a multiple cloning site (MCS) towards the 5' end of the *E. coli lacZ* (α-peptide) gene that enables α-complementation in *E. coli* with the *lacZ*ΔM15 mutation. The MCS is flanked by T3 (5') and T7 (3') RNA polymerase promoters. Expression of inserts cloned into the polylinker in eukaryotes is driven by the cytomegalovirus (CMV) intermediate-early promoter. The *lacZ* sequence 3' of the polylinker is contained within an SV40 intron which lies 5' to an *Herpes simplex* virus thymidine kinase (HSVtk) gene polyadenylation signal. The plasmid is based upon a pBluescript SK(-) ColE1 origin of replication and selection in both prokaryotes and eukaryotes is on the basis of neomycin (kanamycin or G418) resistance. The *neo* gene is expressed under the control of a β-lactamase promoter in bacteria and a simian virus 40 (SV40) promoter in eukaryotes.

pPUR (Clontech) (Fig. 2.2) is an expression vector that contains a 1392bp PvuII/BamHI fragment which encompasses the *Streptomyces alboniger* puromycin N-acetyl transferase (PAC) gene in an SV40 virus early region mammalian expression cassette. This vector provides resistance against puromycin in eukaryotic cells. The plasmid is based upon a pBR322 origin of replication and has ampicillin resistance in bacteria.

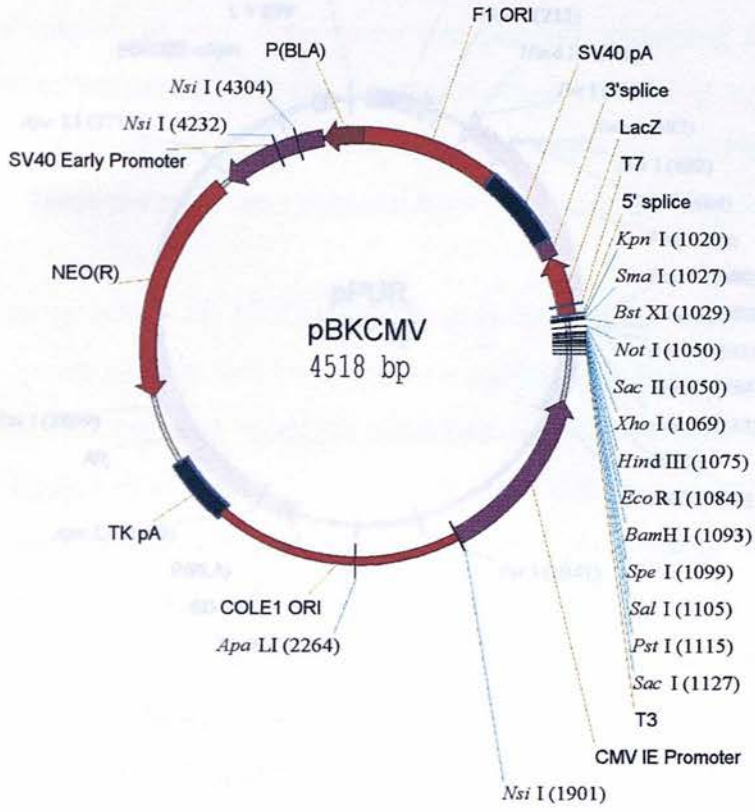


Fig. 2.1. pBK-CMV (Stratagene) is a cloning vector that contains a multiple cloning site towards the 5' end of the E. coli lacZ (α-peptide) gene that enables α-complementation in *E.coli* with the lacZΔM15 mutation. The MCS is flanked by T3 (5') and T7 (3') RNA polymerase promoters. Expression of inserts cloned into the polylinker in eukaryotes is driven by the cytomegalovirus intermediate-early (CMV IE) promoter. The lacZ sequence 3' of the polylinker is contained within an SV40 intron. Selection in both prokaryotes and eukaryotes is on the basis of neomycin (kanamycin or G418) resistance (NEO(R).

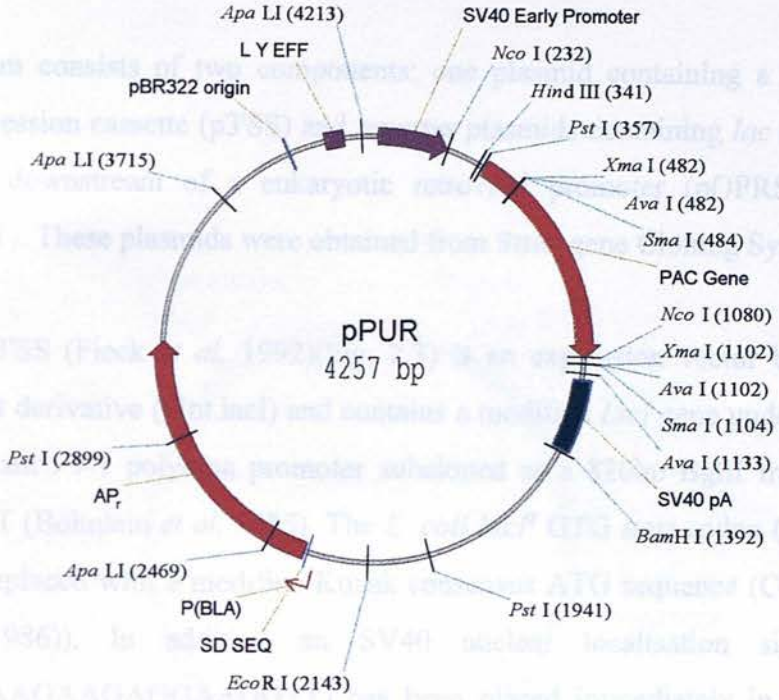


Fig. 2.2. pPUR (also referred to as pBSpacδP) confers puromycin resitance to mammalian cells and can be used to select for stably transformed cell lines. The Streptoyces alboniger puromycin-N-acetyl transferase (PAC) gene has been cloned between the SV40 early promoter and the polyadenylation signals to create a cassette that will express in mammalian cells. Ap_r: prokaryotic ampicillin resitance gene. A: polyadenylation signal. SD SEQ: Shine-Delgarno sequence. P(BLA): prokaryotic β-lactamase promoter

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2.1.2 IPTG-Inducible Expression System Based Upon the *E. coli lac* Operon.

This system consists of two components: one plasmid containing a *lac* repressor (*lacI)* expression cassette (p3'SS) and reporter plasmids containing *lac* operator sites in and/or downstream of a eukaryotic retroviral promoter (pOPRSVICAT and pOPI3CAT). These plasmids were obtained from Stratagene Cloning Systems.

Plasmid p3'SS (Fieck *et al,* 1992)(Fig. 2.3) is an expression vector based upon a pBluescript derivative (pInt.lacI) and contains a modified *lacI* gene under the control of the mutant F9-1 polyoma promoter subcloned as a 820bp BglII fragment from PyF9-1CAT (Bohnlein *et al,* 1985). The *E. coli lacI*^{q} GTG start codon (bases 29-31) has been replaced with a modified Kozak consensus ATG sequence (CCACC**ATG**: (Kozak, 1986)). In addition an SV40 nuclear localisation signal (NLS: CCTAAGAAGAAGAGGAAGGTT) has been placed immediately in front of the *lacI*^{q}stop codon (TGA: bases 1109-1111) but is separated from base 1108 of *lacI*^{q} by a 9bp linker (AGCAGCCTG) and an StuI restriction enzyme site (AGGCCT). The 9bp linker translates to Ser-Ser-Leu and this extends the heptad repeat of the C-terminal leucine zipper motif and thereby enhances the stability of repressor tetramerisation (Fieck *et al,* 1992). An SV40 polyadenylation and small intron from pKO-Neo (Vandoren *et al*, 1984) have been placed downstream of this modified gene. Selection in eukaryotic cells is provided by hygromycin-B resistance gene from COS-203 (Kioussis *et al*, 1987).

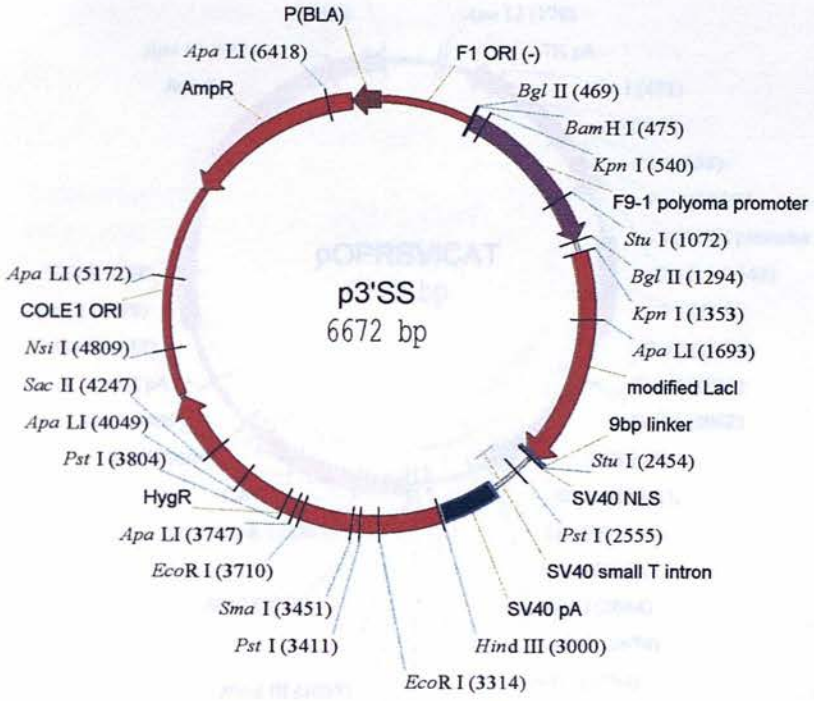


Fig. 2.3. LacI-NLS expression plasmid p3'SS. AmpR: prokaryotic ampicillin resitance gene. PolyA, pA: polyadenylation signal. ORI: prokaryotic or filamentous phage origin of replication. HygR: mammalian hygromycin resitance cassette. F9-1 promoter: from F9-1 polyoma strain. NLS: nuclear localisation signal.

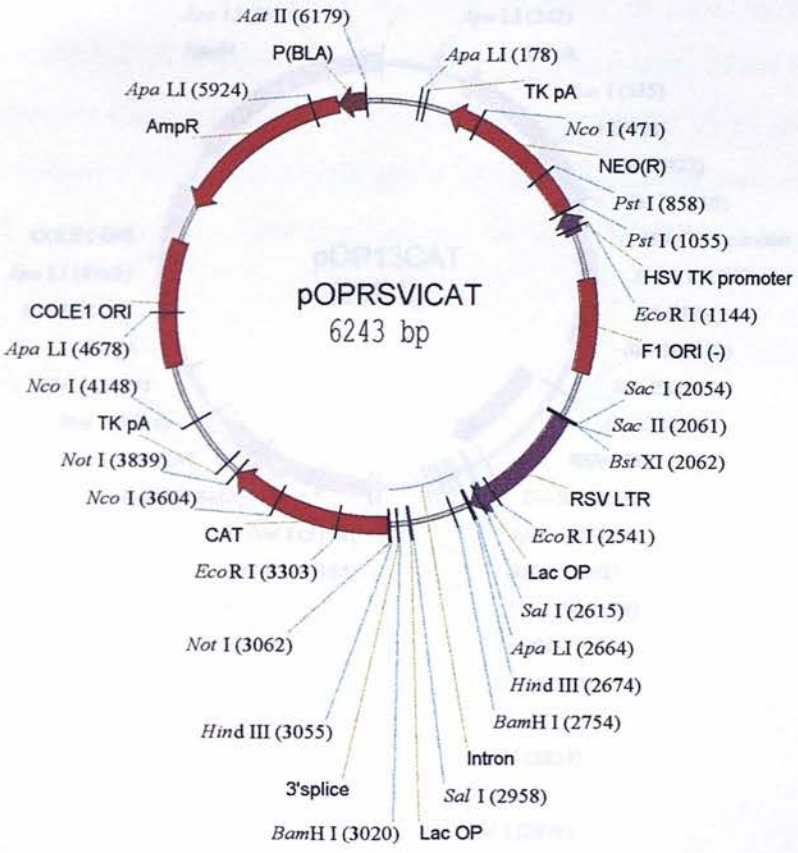


Fig. 2.4. *Lac*-operator-containing mammalian expression vector, pOPRSVICAT. AmpR: prokaryotic ampicillin resistance gene. P(BLA): prokaryotic β-lactamase promoter. HSV TK: Herpes simplex virus thymidine kinase. RSV LTR: Rous sarcoma virus long terminal repeat. TK pA: polyadenylation signal. ORI: prokaryotic or filamentous phage origin of replication. LacOP: ideal lac operator site. NEO (R): mammalian G418 resistance cassette. CAT: chloramphenicol acetyl transferase gene.

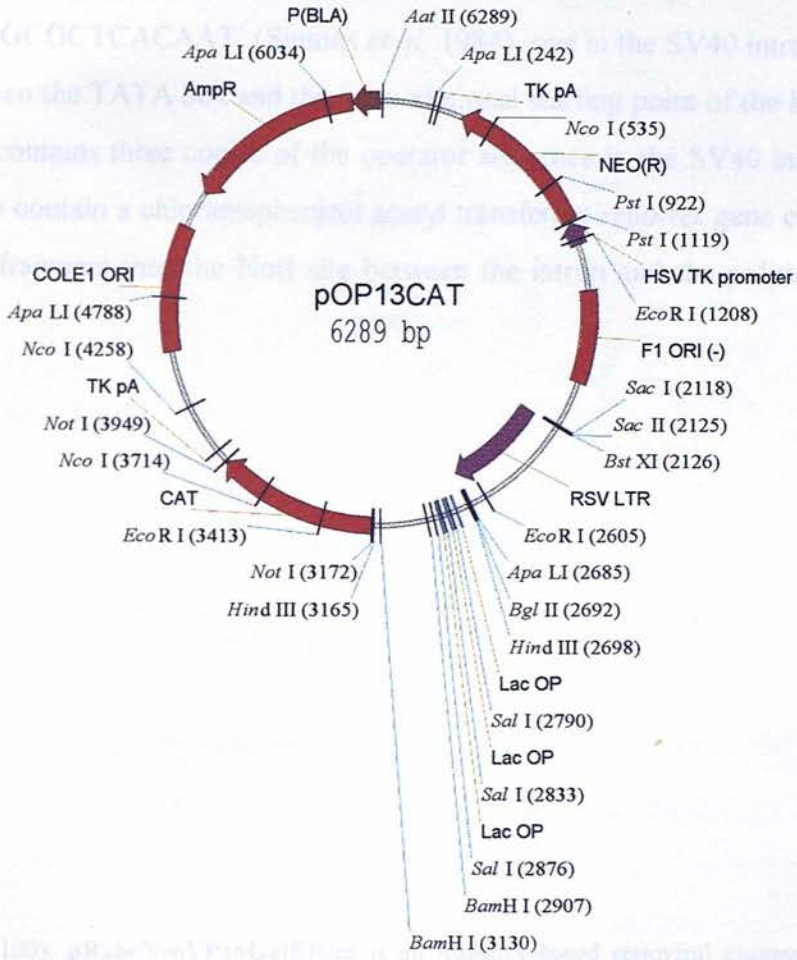
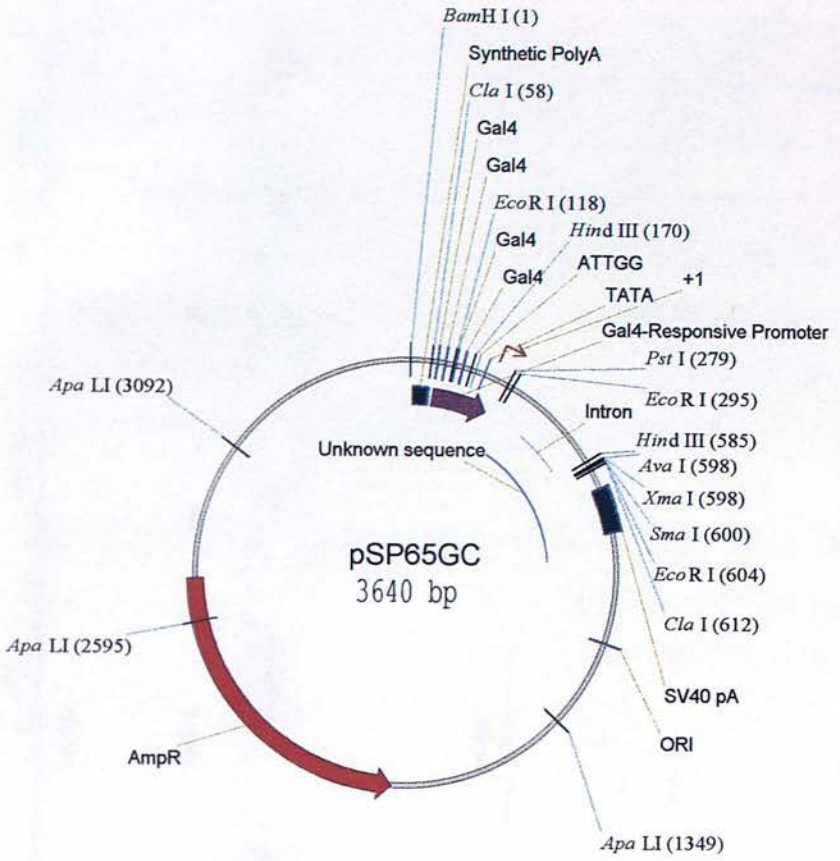


Fig. 2.5. *Lac*-operator-containing mammalian expression vector, pOPI3CAT. (Labels as for pOPRSVICAT).

The *lac*-operator-containing vectors pOPRSVICAT and pOPI3CAT (Figs. 2.4 and 2.5) were constructed from the parental plasmid vector pRSVNot (DuCoeur *et al*, 1992). pRSVNot contains a Rous sarcoma virus long terminal repeat (RSV-LTR) promoter upstream of an SV40 intron a NotI cloning site and an *Herpes simplex* virus thymidine kinase (HSVtk) polyadenylation signal. Selection in bacteria and mammalian cells is by ampicillin and neomycin (G418) resistance respectively. pOPRSVICAT contains two modified ideal *lac* operator sequences (ATTGTGAGCGCTCACAAT: (Simons *et al*, 1984), one in the SV40 intron and one placed between the TATA box and the transcriptional starting point of the RSV-LTR. pOPI3CAT contains three copies of the operator sequence in the SV40 intron. Both plasmids also contain a chloramaphenicol acetyl transferase reporter gene cloned as a 777bp NotI fragment into the NotI site between the intron and the polyadenylation signal.

Fig. 2.7 (Page 100). **pBabeNeoVP16GalERtm** is an MoMSV-based retroviral expression vector containing the VP16galER^{TM} fusion gene.in a 1471bp BamHI/EcoRI fragment. The fusion gene consists of a 237 bp HindIII/SalI fragment from the *Herpes simplex* virus VP16 trans-activation domain (amino acids 416-487), a 223bp SalI/XhoI fragment encoding the DNA binding domain from the yeast Gal4 protein (amino acids 2-74), a 15bp XhoI/SacI/ClaI spacer and a 1162bp ClaI/EcoRI fragment encoding a 4-hydroxytamoxifen-sensitive mutant (G525R: Littlewood *et al*. 1995) of the murine oestrogen receptor steroid binding domain (amino acids 282-595). Resistance in eukaryotes is provided by a G418 resistance cassette. amp^{r}: prokaryotic ampicillin resistance gene. neo^{r}: eukaryotic G418 selection marker. ER^{TM}: murine oestrogen receptor steroid binding domain - tamoxifen mutant. Gal: gal4 DNA binding domain. LTR: Long terminal repeat. MoMSV: Moloney murine sarcoma virus. pA: polyadenylation signal. pUC ori: prokaryotic origin of replication. SV40: SV40 early promoter. VP16: *Herpes simplex* virus VP16 transactivation domain. A: AatII. B: BamHI. Bg: BglII. C: ClaI. E: EcoRI. H: HindIII. N: NotI. Nh: NheI. Pv: PvuI. S: SalII. ScI: SacI. Sca: ScaI. Sp: SpeI. X: XhoI.
Not drawn to scale.



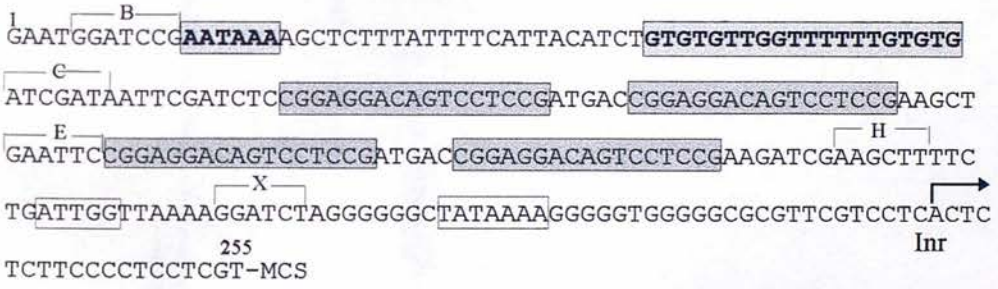
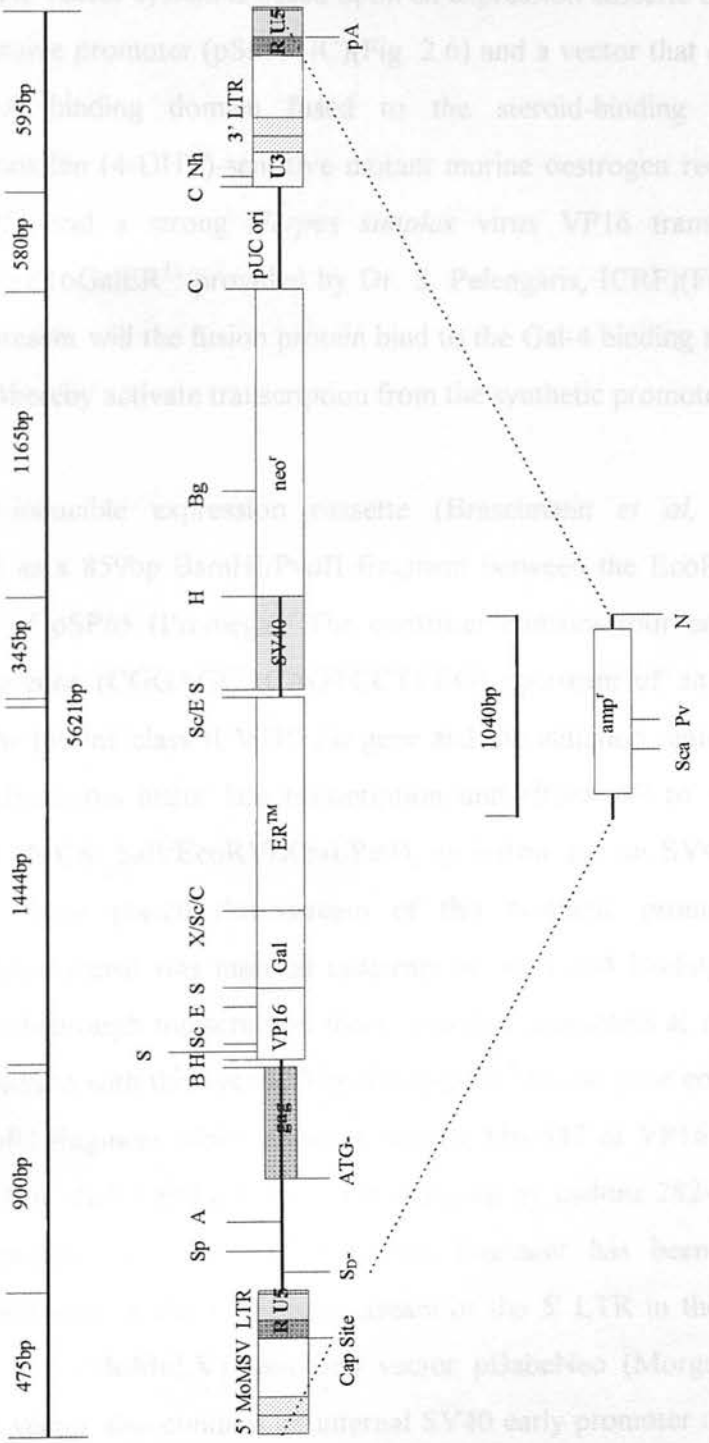


Fig. 2.6. pSP65GC (Top) **and Gal-4 -responsive promoter** (Bottom)(Braselmann al, 1993). AmpR: prokaryotic ampicillin resitance gene. PolyA, pA: polyadenylation signal. Ori: prokaryotic origin of replication. ATTGG: inverted CCAAT box. Inr: initiation region. MCS: multiple cloning site. B: BamHI. C: ClaI. E: EcoRI. H: HindIII. X: XhoII. Xm: XmaIII. Plain boxes: CCAAT and TATA boxes. Bold text in light grey boxes: synthetic polyadenylation signal. Shaded boxes: Gal4 binding sites.


475bp
900bp
1444bp
345bp
1165bp
580bp
595bp
5621bp
5' MoMSV LTR
R U5
Cap Site
Sp
A
Sp
B H Sc E S
S
X/Sc/C
Sc/E S
H
Bg
C
C Nh
3' LTR
R U5
pA
VP16
Gal
ER™
SV40
neo^r
pUC ori
U3
ATG-
1040bp
amp^r
Sca Pv
N

2.1.3 Tamoxifen-Sensitive, Gal4-Responsive Promoter System

This inducible vector system is based upon an expression cassette containing synthetic Gal4-responsive promoter (pSP65GC)(Fig. 2.6) and a vector that expresses the yeast Gal-4 DNA binding domain fused to the steroid-binding domain of a 4-hydroxytamoxifen (4-OHT)-sensitive mutant murine oestrogen receptor (Littlewood *et al,* 1995) and a strong *Herpes simplex* virus VP16 transactivation domain (pBabeNeoVP16GalER^{T}: provided by Dr. S. Pelengaris, ICRF)(Fig.2.7). Only when 4-OHT is present will the fusion protein bind to the Gal-4 binding sites in the reporter vector and thereby activate transcription from the synthetic promoter.

The Gal-4-inducible expression cassette (Braselmann *et al,* 1993) has been constructed as a 859bp BamHI/PvuII fragment between the EcoRI (destroyed) and PvuII sites of pSP65 (Promega). The construct contains four copies of the Gal-4 binding sequence (CGGAGGACAGTCCTCCG) upstream of an inverted CCAAT box from the murine class II MHC *Ea* gene and the initiation region and TATA box from the adenovirus major late transcription unit (from -39 to +15). An multiple cloning site (MCS: SalI/EcoRV/XbaI/PstI), an intron and an SV40 polyadenylation signal have been placed downstream of this synthetic promoter. A synthetic polyadenylation signal was inserted upstream of the Gal-4 binding sites in order to suppress read-through transcription from upstream promoters at the integration site upon transfection with this vector. The VP-Gal-ER^{T} fusion gene consists of a 1444bp BamHI/EcoRI fragment which contains codons 416-487 of VP16 fused in frame to codons 2-74 of Gal-4 and a 15bp linker followed by codons 282-599 of the murine oestrogen receptor (mutant G525R). This fragment has been cloned into the BamHI/EcoRI sites in the MCS downstream of the 5' LTR in the Moloney murine leukaemia virus (MoMuLV) retroviral vector pBabeNeo (Morgenstern and Land, 1990). This vector also contains an internal SV40 early promoter driving a neomycin (G418) resistance gene for selection in mammalian cells.

2.1.4 Plasmids Containing p53 Sequences

Plasmid pMSVcL (Finlay *et al*, 1988) was constructed by digesting pLTRp53cGval135 with XhoI and BamHI to completion and replacing the p53val135 and SV40 sequences with the XhoI/BamHI fragment from p11-4 (Tan *et al*, 1986). The p53 cDNA thus created is identical in sequence to the wild-type p53 clone pp53-17c originally derived and sequenced from an F9 embryonal carcinoma cell line (Pennica *et al*, 1984).

pSV53C contains a full length murine p53 clone constructed from two fragments of cloned cDNA (p1-B and p27.1a) generated from mouse SV3A1E7 polyadenylated mRNA (Jenkins *et al*, 1984). The cDNA is contained within a 1375bp EcoRI/BglII fragment and has four mutations in the non-conserved 5' end of the coding sequence: Arg48 (CGA), Gln79 (CAG), Trp80 (TGG), del81.

2.1.5 Plasmids pMV7 and pMV7-MER

The retroviral expression vector pMV7 (Kirschmeier *et al*, 1988) is based on a derivative of pBR322 which contains a polyomavirus origin of replication as well as retaining the pBR322 origin and ampicillin resistance genes (Dailey and Basilico, 1985; Luskey and Botcham, 1981) It also contains a 4.1kb XhoI fragment encoding a modified Moloney murine sarcoma virus (MoMSV). This MoMSV derivative was produced by removing 3.95kb of MoMSV genomic sequences (coding for the viral *gag* genes and large parts of v-*mos* and the *pol* gene) by digestion with PstI (Perkins *et al*, 1983). It retains both 5' and 3' LTRs and regulatory elements for virion RNA encapsidation and reverse transcription. An HSV*tk*/neomycin phosphotransferase cassette which acts as a dominant selectable marker in eukaryotic cells has been

inserted 0.3kb 5' of the 3' LTR. Two unique cloning sites (EcoR I and Hind III) are found 0.5-0.6kb downstream of the 5' LTR start of transcription.

pMV7-MER (Eilers *et al*, 1989) contains a 2.4kb EcoR I fragment inserted into the unique EcoR I site of pMV7. This fragment consists of a chimaera of cDNA sequences coding for exons II and III of the human c-*myc* protooncogene (Stone *et al*, 1987) fused to cDNA sequences representing the hormone binding domain of the human oestrogen receptor (Kumar *et al*,, 1986).

2.2 Fibroblast Cell Lines

2.2.1 Rat-1A Cells

Rat1A (also known as F2408) is an immortalised, but untransformed, fibroblastoid, 3T3-like cell line spontaneously derived from F344/f (Fischer) rat 19-21 day embryos (Freeman *et al*, 1970 and 1973). It is phenotypically "normal" by various criteria and it is diploid with a modal chromosomal number of 42 (Topp, 1981; Prasad *et al*, 1976). The cells are capable of exponential growth in 1% FCS with a mean doubling time at 37°C of 18 hours, reaching a saturation density of about 2×10^5 cells per cm^2. Cloning efficiency of Rat1 cells is approximately 80% in 10% FCS (Topp, 1981). In transfection experiments, 1-2% of Rat1 cells stained positively for T antigen expression following calcium phosphate precipitation (Wigler *et al*, 1979) with 1μg SV40 viral DNA per 60cm plate (Topp, 1981).

2.2.2 Clone 6 Cells

This cell line (kindly provided by M. Oren) constitutively expresses the temperature-sensitive p53 mutant, p53val135 which adopts wild type conformation and functional properties at permissive temperatures (32°C) and mutant conformation and properties at 37°C (Michalovitz *et al*, 1990). Clone 6 is a cell line derived from low passage rodent embryo fibroblasts transformed by murine p53val135 and a human mutationally-activated c-Harvey-*ras*1 gene (Michalovitz *et al*, 1990) using the calcium phosphate coprecipitation technique (Graham and Van der Eb, 1973). Clones were propagated from foci that developed as a result of co-operation between Ha-*ras* and p53val135 in transformation of the transfected rat embryo fibroblasts at 37°C (Eliyahu *et al*, 1984; Michalovitz *et al*, 1990). Activated Ha-*ras* in these cells is encoded by plasmid pEJ6.6 (Shih and Weinberg, 1982). In Clone 6, p53val135 is under the transcriptional control of a Harvey murine sarcoma virus long terminal

repeat (LTR) and is encoded by plasmid pLTRp53cGval135 (Kaczmarek *et al*, 1986; Eliyahu *et al*, 1985).

2.3 RNA Extraction

2.3.1 Treatment of Water and Equipment for use with RNA

Batches of deionised distilled water (DDW) were prepared for use in RNA work by treating with diethyl pyrocarbonate (DEPC) at a concentration of 0.1% overnight in a fume cupboard and were then autoclaved.

Similarly, items of equipment for use with RNA such as electrophoresis tanks, forceps, glass dishes and slot-blot apparatus were treated with DDW containing 0.1% DEPC overnight. Equipment was then autoclaved or rinsed thoroughly with diethyl pyrocarbonate-treated deionised distilled water (DEPC/DDW).

2.3.2 Isolation of Total RNA

Total RNA was isolated from mouse tissues or from cell cultures using a monophasic solution of guanidine isothiocyanate and phenol (TRIzol, Life Technologies) in a modified Chomczynski and Sacchi (1987) protocol. Autoclaved plasticware and filter tips were used to prevent RNA degradation by contaminating RNAses. Stock solutions and pipettes for RNA work were kept separate from those used for other purposes.

Tissues were removed from freshly killed mice and promptly snap frozen in liquid nitrogen and stored at -70°C until required for RNA extraction. Approximately 100mg of tissue was homogenised in 1ml TRIzol solution and incubated at room temperature for 5 minutes before vigorously mixed with 200µl chloroform (Sigma).

The mixture was incubated at room temperature for 3 minutes and then centrifuged at 6500 rpm at 4°C in an MSE MicroCentaur centrifuge. The aqueous phase was then carefully removed and total RNA was precipitated with 0.5 ml isopropanol (propan-2-ol, Sigma) at room temperature for 10 minutes. The precipitated total RNA was collected by centrifugation at 13000 rpm for 10 minutes at 4°C and washed once with 70% ethanol in DEPC/DDW. The pellet was dried *in vacuo* for 3-4 minutes and then resuspended in 100µl DEPC/DDW. The concentration of total RNA was estimated by ultraviolet spectrophotometry at 260nm using a Genequant II RNA/DNA Calculator (Pharmacia). RNA samples were stored at -70°C until required.

RNA from cell cultures was prepared directly from cells grown as monolayers on 10cm tissue culture plates (Nunc). The cells were washed twice with 5 ml phosphate buffered saline (PBS) and lysed *in situ* with 1 ml TRIzol solution. Isolation of total RNA from the TRIzol lysates was carried out as above except that RNA pellets were resuspended in 50µl DEPC/DDW.

2.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.4.1 Reverse Transcription

First strand complementary DNA (cDNA) was synthesised from total RNA using an RNAse H- Moloney Murine Leukaemia Virus (MoMuLV) Reverse Transcriptase (Superscript II, Life Technologies) and an oligo(dT) primer. 1-2 µg of total RNA was heated with 100ng primer $p(dT)_{10}$ (Boehringer Mannheim) to 70°C for 5 minutes and plunged on ice for a further 5 minutes. A reaction mixture containing 4µl 5X First Strand Buffer, 1µl 0.1M dithiothreitol (DTT), 200 Units RNAse H- Superscript and 1µl 10X mixed dNTP stock solution (Appendix A) was prepared. This reaction was then incubated on ice for 10 minutes before being placed on a thermal heating block

(Omni-gene, Hybaid) on the following programme: 37°C for 10 minutes, 1 hour at 42°C, 10 minutes at 50°C and 10 minutes at 94°C to inactivate the reverse transcriptase. Control reactions were carried out to which no RNA was added. cDNAs were diluted to 100µl with DDW and stored at -20°C.

2.4.2 RT-PCR for Human c-Myc

Total RNA from the A549 human lung epithelial cell line was isolated and reverse transcribed as described above and subjected to PCR with oligodeoxyribonucleotide primers: 5'-CCGAATTCGACGATGCCCCT-3' (5987: myc5') and 5'-GGCCGCTTACGCACAAGAGTT-3' (3212: myc3'). Both primers were custom-manufactured and cartridge-purified by Cruachem Ltd.

The PCR reaction mixture contained 5µl of diluted A549 cDNA, 10µl 10X Thermophilic Buffer, 6µl 25mM magnesium chloride, 3 Units *Taq* (*Thermus aquaticus)* DNA polymerase (Promega), 2µl mixed dNTP stock solution (Appendix A) and 100ng of each primer made up to a final volume of 100µl with autoclaved DDW. The mixture was overlaid with 75µl paraffin oil (Boots) and amplification was carried out on a thermal cycler (OmniGene, Hybaid) on the following programme:

Step1: 94°C, 3 minutes,
55°C, 1 minute,
72°C, 2½ minutes; once.

Step 2: 55°C, 30 seconds,
72°C, 2½ minutes,
94°C, 30 seconds; repeated 34 times.

Step 3: 60°C, 1 minute,
72°C, 5 minutes,
30°C, 30 seconds; once.

10µl of RT PCR products were analysed on a 1% agarose gel in 1X TBE as described below. Products were stored at -20°C.

2.4.3 RT-PCR for the Mouse Nedd2 Open Reading Frame

Total RNA from 129/Sv mouse liver was isolated and reverse transcribed as described above and subjected to PCR with oligodeoxyribonucleotide primers: 5'-AAATGGCGGCGCCGAGCGGGAGGTCGCAGT-3' (3444: Nedd2 5') and 5'-GGCGGCATCACGTGGGT-3' (3445: Nedd2 3'). Both primers were custom-manufactured and purified by High Performance Liquid Chromatography (HPLC) by Cruachem Ltd.

The PCR reaction mixture contained 20µl of diluted mouse liver cDNA, 10µl 10X Thermophilic Buffer, 6µl 25mM magnesium chloride, 3 Units *Taq* (*Thermus aquaticus)* DNA polymerase (Promega), 2µl mixed dNTP stock solution (Appendix A) and 100ng of each primer made up to a final volume of 100µl with autoclaved DDW. The mixture was overlaid with 75µl paraffin oil (Boots) and amplification was carried out on a thermal cycler (OmniGene, Hybaid) on the following programme:

Step1: 94°C, 3 minutes,
55°C, 1 minute,
72°C, 2½ minutes; once.

Step 2: 55°C, 30 seconds,
72°C, 2½ minutes,
94°C, 30 seconds; repeated 34 times.

Step 3: 60°C, 1 minute,
72°C, 5 minutes;
30°C, 30 seconds; once.

10µl of RT PCR products were analysed on a 1% agarose gel in 1X TBE. Products were stored at -20°C.

2.4.4 RT-PCR assay for *nedd2* mRNA Expression in Transfected Cells.

Total RNA from transfected Rat-1 fibroblasts was isolated, treated with Amplification-Grade DNase-I (Life Technologies) according to the manufacturer's instructions and reverse transcribed as described above and subjected to PCR with oligodeoxyribonucleotide primers: 5'-AGCATGTCGTGGAGATGAGA-3' (N5) and 5'-GGCGGCATCACGTGGGT-3' (3445: Nedd2 3'). Both primers were custom-manufactured and purified by High Performance Liquid Chromatography (HPLC) by Cruachem Ltd.

The PCR reaction mixture contained 20µl of diluted (1 in 10) DNase-treated cDNA, 4% DMSO, 5µl 10X Thermophilic Buffer, 3µl 25mM magnesium chloride, 1.25 Units *Taq* (*Thermus aquaticus)* DNA polymerase (Promega), 2µl mixed dNTP stock solution (Appendix A) and 100ng of each primer made up to a final volume of 50µl with autoclaved DDW. The mixture was overlaid with 75µl paraffin oil (Boots) and amplification was carried out on a thermal cycler (OmniGene, Hybaid) on the following programme:

Step1: 94°C, 3 minutes,
55°C, 1 minute,
72°C, 2 minutes; once.

Step 2: 55°C, 30 seconds,
72°C, 30 seconds,
94°C, 30 seconds; repeated 34 times.

Step 3: 60°C, 1 minute,
72°C, 5 minutes;
30°C, 30 seconds; once.

10µl of RT PCR products were analysed on a 1% agarose gel in 1X TBE. Products were stored at -20°C.

2.4.5 RT-PCR for Murine p21$^{WAF1/CIP1}$ (Second Exon)

Total RNA from 129/Sv mouse liver was isolated and reverse transcribed as described above and subjected to PCR with oligodeoxyribonucleotide primers 5'-CAGGATCCATGCCAATCCTGGT-3' (G5650: WAF5') and 5'-TCGGTACCTGTCAGGCTGGT-3' (G5649: WAF3'). Both primers were custom-manufactured and purified by HPLC by Oswel DNA Service.

The PCR reaction mixture contained 5µl of diluted mouse liver cDNA, 10µl 10X Thermophilic Buffer, 6µl 25mM magnesium chloride, 5µl dimethyl sulphoxide (DMSO), 3 Units *Taq* (*Thermus aquaticus)* DNA polymerase (Promega), 2µl mixed dNTP stock solution (Appendix A) and 100ng of each primer made up to a final volume of 100µl with autoclaved DDW. The mixture was overlaid with 75µl paraffin oil (Boots) and amplification was carried out on a thermal cycler (OmniGene, Hybaid) on the following programme:

Step1: 94°C, 3 minutes,
55°C, 1 minute,
72°C, 2½ minutes; once.

Step 2: 55°C, 30 seconds,
72°C, 1 minute,
94°C, 30 seconds; repeated 34 times.

Step 3: 60°C, 1 minute,
72°C, 5 minutes,
30°C, 30 seconds; once.

10µl of RT PCR products were analysed on a 2% agarose gel in 1X TBE as described below. Products were stored at -20°C.

2.4.6 High Fidelity RT-PCR for Murine p21$^{WAF1/CIP1}$

High Fidelity amplification was carried out for two oligodeoxyribonucleotide primer sets: one set, WAF5' and WAF3', produce a 446bp product corresponding to codons 1-143 of p21 cDNA and the other set, WAF5' and WAF23 (15027, 5'-GGCACTTCAGGGTTTTCTCTTGC-3': Cruachem Ltd) amplifies the full ORF encoding p21 and includes the stop codon (codon 160; underlined).

Total RNA from 129/Sv mouse liver was isolated and reverse transcribed as described above and subjected to high fidelity PCR with either of the primer sets. High Fidelty PCR was achieved using a mixture of conventional *Taq* (*Thermus aquaticus)* DNA polymerase and *Pwo* (*Pyrococcus woesei*) DNA polymerase (Expand High Fidelity, Boehringer Mannheim). In addition to the 5'-3' polymerase activities of both thermostable polymerases, this mixture takes advantage of the proofreading 3'-5' exonuclease activity of *Pwo* to provide a 4 fold lower error rate (8.5×10^{-1}) as compared to *Taq* (2.6×10^{-1}). Expand High Fidelity PCR products consist of mixtures of molecules with 3' adenosine overhangs and blunt-ended fragments and are thus suitable for use in T/A cloning procedures.

For Expand High Fidelity PCR, two mastermixes were made so that template and nucleoside triphosphates were kept separate until the latest possible moment prior to denaturation in order to prevent degradation of the template and nucleosides by the exonuclease activity of *Pwo*. Mastermixes were made large enough to allow for 6 reactions, one additional reaction with control reverse transcription product (no RNA) and an extra reaction to allow for errors in pipetting. For each reaction, Mastermix A contained 2µl dNTP stock solution, 200ng of each primer from one of the above primer sets, 5µl diluted cDNA and 41µl DDW. Mastermix A was then dispensed in 50µl aliquots into 0.5 ml microcentrifuge tubes and 1,2,3,4,5 or 6µl

dispensed in 50µl aliquots into 0.5 ml microcentrifuge tubes and 1,2,3,4,5 or 6µl 25mM magnesium chloride added and made up to 56µl total volume with DDW. For each reaction Mastermix B contained 10X magnesium free PCR buffer, 0.75µl Expand enzyme mix and DDW to a final volume of 44µl. Prior to amplification, 44µl of Mastermix B was mixed with the 56µl aliquots of Mastermix A containing cDNA and magnesium chloride and overlaid with 75µl paraffin oil (Boots). Amplification was carried out on a thermal cycler (OmniGene, Hybaid) on the following programme:

Step1: 94°C, 2 minutes, once.
Step 2: 94°C, 45 seconds,
55°C, 1 minute,
72°C, 1 minute; repeated 10 times.
Step 3: 94°C, 45 seconds,
55°C, 45 seconds,
72°C, 1 minute + 20 seconds increment per cycle; repeated 20 times.
Step 4: 72°C, 10 minutes; once.

10µl of RT PCR products were analysed on a 1% agarose gel in 1X TBE as described below. Products were stored promptly at -20°C.

2.5 General Plasmid DNA Analytic and Preparative Techniques.

2.5.1 Restriction Endonuclease Digestion of Plasmid DNA

Plasmid DNA was digested in sterile microfuge (Eppendorf) tubes containing a mixture of DDW, the appropriate restriction endonuclease and the recommended, correct ionic strength buffer. All restriction enzymes were supplied by Boehringer Mannheim, Promega or Stratagene.

10 X stock buffer solutions were used diluted 1:10 in the final reaction mix. Restriction enzymes were stored at -20^{o}C in buffers containing 50% glycerol and kept on ice while in use. Sufficient enzyme was used to ensure complete digestion: 1 unit of enzyme being the amount required to digest 1μg of pBR322 plasmid DNA to completion in 1 hour. The amount of enzyme was kept below 10% of the final reaction volume as glycerol can interfere with enzymatic activity. Digestion was carried out at 37^{o}C for most enzymes for a minimum of 1 hour.

2.5.2 Agarose Gel Electrophoresis of DNA

Agarose gel electrophoresis was carried out in order to confirm digestion, characterise plasmids or purify restriction fragments. DNA molecules are separated by electrophoresis according to size, conformation (supercoiled, nicked circular or linear), agarose concentration and applied current. Tris-borate EDTA (TBE: appendix A) was routinely used as an electrophoresis buffer. Preparation of gels and electrophoresis in BioRad MiniSub Cells or Anachem Origo H3 gel tanks was carried out as described by Sambrook *et al,* (1989). Samples were loaded by adding 0.1

volume gel loading buffer (Appendix A) and applied to the wells of a gel submerged in electrophoresis buffer. Fragment sizes were estimated with reference to a kilobase marker (Life Technologies) or Marker V (Boehringer Mannheim).

2.5.3 Isolation of DNA Fragments from Agarose Gels

Digested DNA fragments visualised by UV transillumination were excised from 0.8%-1% agarose / TAE buffered (appendix A) gels and purified using the GlassMAX DNA Isolation Spin Cartridge System (Life Technologies) according to the manufacturer's instructions.

2.5.4 Phenol:Chloroform Extraction and Ethanol Precipitation of DNA

These procedures were carried out as described in Sambrook *et al*, (1989) in order to remove proteins and to concentrate DNA. The resulting DNA pellets were then briefly air-dried and redissolved in an appropriate volume of TE (pH 8.0: appendix A).

2.5.5 Estimation of DNA Concentration and Purity

Two methods for the estimation of the concentration of DNA solutions were used: a spectrophotometric method and an ethidium bromide fluorescent quantitation method (Sambrook *et al*, 1989). DNA samples from large and small scale DNA preparations were quantitated by ultraviolet spectrophotometry.

The optical densities (OD) of 1:100-diluted DNA solutions were measured in a Genequant II RNA/DNA Calculator (Pharmacia) at λ=260nm. An of OD_{260} of 1 was taken to be equivalent to a concentration of 50µg/ml of double-stranded DNA in the diluted sample. The ratio of the ODs at λ=260nm and at λ=280nm is a measure of the

purity of a DNA sample. Accordingly, $OD_{260\,280}$ ratios measured in the Genequant were recorded for DNA samples.

The concentrations of certain DNA solutions (such as the eluates from GlassMAX columns) were estimated by an ethidium bromide fluorescence method. Small samples of DNA (eg. 0.5µl) and a range of serially-diluted kilobase ladder were spotted onto a plastic Petri dish containing 1% agarose gel and 5µg/ml ethidium bromide. After 10-15 minutes to allow absorption of DNA onto the gel, the plate was exposed on a ultraviolet transilluminator and the DNA concentration was estimated by comparison of the fluorescent intensity of the sample against that of the standards.

2.5.6 Modification of the Ends of linear DNA molecules: Blunt-Ending

Restriction fragments of plasmids were blunt-ended by addition of 0.1 volume 10mM dNTP stock solution and 1µl T4 DNA polymerase directly to a completed restriction reaction mix and incubated at 37°C for 30 minutes. T4 DNA polymerase blunt-ends both 5' and 3' overhangs effectively as it has an efficient 3'-5' exonuclease activity as well as its 5'-3' polymerase activity. The enzyme was inactivated by incubation at 65°C for 15 minutes.

2.5.7 Modification of the Ends of Linear DNA Molecules: Linker Addition

2µg Blunt-ended linear DNA molecules were ligated with 1µg of phosphorylated synthetic linkers containing an appropriate restriction enzyme recognition sequence. 1µl of linker (1µg), 6 units T4 DNA ligase and 0.1 volume 10X Ligase Buffer were added to a heat-inactivated restriction enzyme (and blunt-ending) reaction mix and ligated overnight at 4°C. In the case of restriction reactions that could not be inactivated by heat, linkers were ligated to blunt-ended fragments that had been phenol/chloroform extracted and ethanol precipitated.

Following ligation, the ligase was inactivated by heating to 65°C for 15 minutes and the linkers were digested by the addition of 30 units of the appropriate restriction enzyme and incubated at 37°C for at least 4 hours. If necessary, the buffer conditions were altered to those optimal for the restriction enzyme. The digested reaction mix was then run on a TAE gel and the appropriate band recovered and purified. The purified bands contained compatible cohesive termini of the appropriate restriction enzyme.

2.5.8 Modification of the Ends of Linear DNA Molecules: Dephosphorylation

In a ligation reaction in which the vector has compatible or blunt ends, 5' dephosphorylation of the vector fragment considerably reduces the number of background non-recombinants that can be transformed into *E. coli*. This means that there is a strong chance that in a small number of colonies (6-12) screened by small scale DNA preparations, the desired recombinant molecule can found. Accordingly, wherever possible, gel-purified vector fragments were treated with either calf-intestinal alkaline phosphatase (CIAP: Promega) or arctic shrimp alkaline phosphatase (SAP: United States Biochemicals) prior to use in ligation reactions. SAP has the advantage that it is easily inactivated by heating to 65°C for 15 minutes, wereas CIAP is more resistant to heat and needs to be eliminated by phenol/chloroform extraction and ethanol precipitation.

1 unit SAP or CIAP was added to 40µl gel-purified vector fragment together with the appropriate dephosphorylation buffer and DDW up to a final volume of 50µl. This reaction was incubated at 37°C for 1 hour and then the phosphatase was inactivated using the appropriate method. Vector fragments were quantitated by the ethidium bromide plate method and used in ligation reactions.

2.5.9 T/A Cloning of RT-PCR Products into Plasmid Vectors

RT-PCR products were cloned by ligation into the pGEM-5Zf(+) plasmid vector linearised at an EcoRV site. This site is modified so that it possesses 3'-A overhangs in order to complement 5'-T overhangs on PCR products (pGEM-T Vector System, Promega). 1µl of PCR product was ligated to 50ng pGEM-T vector in a 10µl ligation reaction containing 3 units T4 DNA ligase and 1X Ligase Buffer (Promega; Appendix A) at 15°C overnight. 1µl of the reaction was transformed into XL1-BlueMRF' competent cells (Stratagene; see below) and plated onto L-Amp plates that had been treated with 100µl 20mM IPTG (isopropyl-β-D-thio-galactopyranoside; Stratagene) and 100µl 0.5% X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside: Boehringer Mannheim) for 30 minutes at 37°C (L-Amp/X-Gal/IPTG plates). Plates were incubated overnight at 37°C. White (or light blue) colonies indicated recombinant plasmids containing cloned PCR product or having a *lacZ* α-peptide reading frame that was disrupted by some other mechanism.

2.5.10 Sub-Cloning of Gel-Purified DNA Fragments Between Plasmid Vectors

Plasmid DNA was digested with the appropriate restriction enzyme(s) in order to release a desired fragment to be subcloned or to linearise a vector prior to ligation. 2µg of plasmid was cut for production of vector or 5µg of plasmid was cut in order to release a fragment for subcloning. If modification of fragment ends by blunt-ending or linker addition was required, this was carried out at this point. The digested (and modified) fragments were then gel-purified as described above. Vector fragments were then dephosphorylated in order to limit recircularisation and therefore non-recombinant frequencies. Usually three ligation reactions were carried out per experiment. One control reaction contained only vector fragment, while the other experimental reactions contained a 1:1 or a 2:1 molar ratio of insert to vector respectively. The mass of vector was kept constant (50ng) and the amount of insert required for a 1:1 ratio was determined using the following formula:

$$\frac{50\text{ng (mass of vector)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} = \text{mass of insert (ng).}$$

The vector and insert were ligated in a 10µl ligation reaction containing 3 units T4 DNA ligase and 1X Ligase Buffer (Promega; Appendix A) at 15°C overnight. 1µl of the reaction was transformed into the appropriate strain of competent cells.

2.5.11 Transformation of Competent *Escherichia coli* with Plasmid DNA

Circularised plasmid DNA was introduced into commercially produced competent *E. coli* of the appropriate strain (eg. XL1BlueMRF' competent cells: Stratagene. For more information on strains see Appendix B) according to the following protocol:

Competent cells were defrosted on ice and aliquoted into pre-chilled 1.5 ml microfuge tubes. 1µl of undiluted ligation reaction was gently mixed with 20µl of competent cells and incubated on ice for 20 minutes. Cells were then heat-shocked at 42°C for 40 seconds and plunged on ice for 2 minutes. Following the addition of 80µl of room temperature SOC medium (Appendix A) the cells were incubated at 37°C in an orbital incubator at 220 rpm for 1 hour to allow expression of antibiotic resistance.

Transformed cells were innoculated onto L-amp plates (LB agar with 100µg/ml ampicillin: Appendix A) or onto plates containing 50µg/ml Kanamycin Sulphate (Sigma) in the case of bacteria containing pBKCMV-derived vectors. Plates were incubated at 37°C for 16-24 hours. Controls were provided by parallel transformations with pUC19 DNA (positive control) or no DNA (negative control) plated onto L-amp plates or bacteria treated as above but with no DNA plated onto LB plates (positive control: no antibiotic)

2.5.12 Identification of Recombinant Plasmids by Colony Hybridisation

Recombinant clones were identified by colony hybridisation if there was a large number of non-recombinant clones on the bacterial plate corresponding to the control (0:1) ligation. Colonies were replica-plated onto duplicate L-Amp plates, one having a gridded nylon filter (Hybond N+: Amersham) in contact with the agar. After overnight incubation at 37°C, the master plates were stored at 4°C and the gridded filters were processed according to a protocol modified from Sambrook *et al*, 1989. Briefly, filters were placed colony side up on 0.75 ml 0.5N NaOH on a piece of Clingfilm for 3 minutes. The underside of the filters were blotted on a paper towel and were treated again with NaOH and blotted. The filters were neutralised on two 5 minute changes of 1M Tris (pH7.4) followed by one change of 1.5M NaOH, 0.5M Tris (pH7.4). The filters were allowed to dry on a piece of 3MM paper for 20-30 minutes and the bacterial DNA was UV-crosslinked to the filters (Spectrolinker XL1500: Spectronics Corporation). Filters were stored dry between pieces of 3MM paper until required for hybridisation. Hybridisation was carried out as detailed below and recombinant colonies were identified on the master plates by alignment with the autoradiographs.

2.5.13 DNA/DNA Hybridisation

DNA probe fragments were gel purified and 20ng labelled with 50μCi, 3000Ci/mmol [α-^{32}P]-dCTP (ICN) using the Prime-It RmT random primer labelling kit (Stratagene). Unincorporated nucleotides were removed by passing labelled probes down a Sephadex G50 column (NICK Column: Pharmacia).

Filters were prehybridised in a hybridisation oven (Hybaid) for at least 4 hours at 65°C in Hybridisation Buffer (Appendix A). Labelled DNA probe was denatured by boiling and then immediately diluted in hybridisation solution at 65°C and the prehybridised filters were hybridised at 65°C with the probe overnight.

The hybridisation solution was discarded and the filters washed once in 100ml 2X SSC (Appendix A), 65°C for 30 minutes; once in 100ml 2x SSC, 1% SDS, 65°C for 30 minutes; once in 100ml 0.5X SSC, 1% SDS, 65°C for 30 minutes and once in 100ml 0.1X SSC at room temperature for 30 minutes. The filters were sealed moist in heat-sealable plastic bags and exposed to Fuji R-X film at -70°C in an autoradiography cassette with intensifying screens.

2.5.14 Small-scale Preparation of Plasmid DNA (Mini-preps)

Isolation of plasmid DNA from bacterial cultures for the verification of plasmid identity by restriction digestion was carried using the Wizard Minipreps DNA Purification System (Promega). Small-scale preparations suitable for manual sequencing were performed using the QIAprep Spin Plasmid Kit (Qiagen). Both systems were used to purify plasmid DNA from cleared bacterial alkaline SDS lysates using high concentrations of chaotropic salts (guanidine hydrochloride or guanidinium isothiocyanate) and a silica gel-based DNA-binding resin (Wizard kit) or membrane (QIAprep kit). The DNA on the mini-columns was washed with ethanol containing high-salt solutions and eluted in low salt (TE) buffer.

Preparation of cleared bacterial lysates for use with the Wizard columns is described as an example below; the procedure for the QIAprep system is similar and will not be detailed here. The plasmid DNA was purified from cleared lysates with the kits in accordance with the manufacturers' instructions.

Single colonies were picked from L-amp plates and innoculated into 5mls LB medium containing the appropriate antibiotics (100µg/ml ampicillin or 50µg/ml kanamycin) in Falcon 2059 tubes (Becton Dickinson). After an incubation period of 14-16 hours at 37°C in an orbital incubator at 220 rpm, a 1.5ml sample (3ml for QIAprep) from each of the resulting late logarithmic phase cultures was transferred to a microfuge tube and the bacteria pelleted by microcentrifugation at 6500 rpm for 5 minutes. The

supernatants were discarded and the bacterial pellets resuspended in 200µl Cell Resuspension Buffer (Appendix A), lysed by the addition of 200µl of an alkaline sodium dodecyl sulphate solution (SDS)(Cell Lysis Buffer: appendix A. The SDS denatures bacterial proteins and the strong alkaline conditions used in this procedure cause denaturation of both plasmid and chromosomal DNA) and gently mixed by inversion. The mixture was then neutralised with 200µl Neutralisation solution (Appendix A). This neutralisation caused rapid renaturation of circular plasmid DNA wereas most chromosomal DNA, bacterial protein and SDS (as potassium dodecyl sulphate) formed a white gelatinous precipitate which was separated from the aqueous, plasmid-containing supernatant upon microcentrifugation at 13000 rpm for 5 minutes. This supernatant (cleared lysate) was promptly separated from the debris by pipetting into clean microfuge tubes.

1-2µl samples of DNA purified from cleared lysates were digested with appropriate restriction enzymes and analysed by agarose gel electrophoresis (2.3.1).

2.5.15 Large-scale Preparation of Plasmid DNA (Maxi- and Mega-preps)

This method is a larger scale version of the alkaline lysis procedure described above and rapidly prepares large amounts DNA of a quality that is suitable for ligation, sequencing and transfection. The DNA from the bacterial lysates is highly purified by anion-exchange column chromatography (Qiagen Tip-100 or Tip-2500, Qiagen).

Cleared bacterial lysates were prepared according to the column manufacturer's instructions from a 100ml (maxi-prep: Tip-100) or a 500ml (mega-prep: Tip-2500) overnight culture (no longer than 16 hours incubation) and then decanted through stainless-steel strainers into 50ml polypropylene universal containers and applied to equilibrated (with QBT buffer: Appendix A) Qiagen columns. The plasmid DNA was washed with the supplied wash buffer (QC: Appendix A), eluted in a buffer containing 1.25M NaCl at pH 8.5 (QF: Appendix A) into clean Oakridge centrifuge tubes and

precipitated with 0.7 volume room temperature isopropanol. The DNA was collected by centrifugation at 11500rpm in a Sorval SS-34 rotor and washed with a small volume of ice-cold 70% ethanol. After removal of excess ethanol, the pellets were dried *in vacuo* for no more than 5 minutes before being resuspended in an appropriate volume of sterile TE (Appendix A). The concentration and purity of the DNA was estimated as above.

2.5.16 Dideoxynucleotide Chain-Termination (Sanger) Sequencing of Plasmid DNA

Double-stranded plasmid DNA template was prepared by the QIAprep Spin mini-prep or Qiagen maxi-prep methods and then alkaline-denatured. For each sample, 5µg DNA was diluted in 45µl DDW and denatured with 5µl 2M NaOH, 2mM EDTA and incubated at ambient temperature for 5 minutes. The solution was neutralised with 2M ammonium acetate (pH4.6) and the DNA precipitated at -70°C for 30 minutes with 185µl absolute ethanol. The DNA was recovered by microcentrifugation at 13000 rpm at 4°C for 10 minutes and the pellet was washed once with ice-cold 70% ethanol and dried *in vacuo* for 5 minutes. The DNA was either resuspended in 6µl DDW immediately for further processing or was stored dry at -20°C for up to one week.

The resuspended DNA was annealed to 2pmol primer in 1X Sequenase Reaction Buffer (United States Biochemicals) in a final volume of 10µl by heating to 65°C for 2 minutes and then allowed to cool to 25°C, slowly (over 15-30 minutes) in a waterbath placed on the bench. The annealed template/primer mix was then labelled at ambient temperature for 4 minutes with 5µCi, (1000Ci/mmol)[α-^{35}S]-dATP (ICN) and terminated at 37°C for at least 5 minutes with each of the four dideoxyribonucleotides using dGTP reagents and protocols provided with the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemicals). Each termination reaction was stopped using the provided 'Stop' solution which also acts as a dye-containing loading buffer. Samples were stored at -20°C until required.

Processed samples (termination reactions) were denatured by heating to 75°C immediately before loading 3µl into wells created by a shark's tooth comb at the top of a pre-run (45°C) 6% denaturing polyacrylamide sequencing gel (Appendix A). The gel was poured between two glass plates separated with 0.4mm plastic spacers. One plate was coated with silicone (Gel-Slick) to prevent adhesion of the gel to both plates. The gel was run using S2 sequencing apparatus (Life Technologies) and a model 3000 microcomputer electrophoresis power supply (Life Technologies) at 70W in 1X TBE.

Following running of a sequencing gel, the siliconed plate was removed and the gel was fixed with two 4 minute changes of 10% methanol, 10% acetic acid and transferred to a piece of 3MM paper. The gel was covered in plastic film (Clingfilm) and dried *in vacuo* for 2 hours at 80°C using a model 583 gel dryer (BioRad) and vacuum pump. The Clingfilm was removed and the gel was exposed to Biomax MR film (Kodak) in an autoradiography cassette for 18-48 hours. The film was then processed in a processor (Hyperprocessor: Amersham) and viewed on a white light box.

2.5.17 Preparation of Uni-directional Nested Deletions in pBK53wt DNA.

Uni-directional nested deletions were made in plasmid pBK53wt so that sequencing of the entire insert could be performed using only one sequencing primer. The method exploits the highly predictable rate of digestion by exonuclease III of double-stranded DNA to single-stranded DNA in the 3'-5' direction to produce a uniform series of progressively shorter DNA fragments. Exonuclease III does not digest the 3' ends of single-strand extensions or a 5' restriction overhang that has been filled in with α-thio dNTPs and therefore can made to digest only one arm of a doubly-digested plasmid. Mung bean nuclease digestion removes the remaining overhangs and the resulting blunt-ended DNA is then recircularised with DNA ligase and transformed into *E. coli*.

The T3 sequencing primer in pBK53wt was protected from deletion by exonuclease III by cutting 60μg plasmid with SalI in a 500μl reaction and filling in the 5' overhangs with 5 units Klenow fragment of *E.coli* DNA polymerase I in the presence of 4μM α-thio dNTPs (Stratagene) at ambient temperature for 15 minutes. After phenol/chloroform extraction and ethanol precipitation the DNA was cut with EcoRI in a 500μl reaction and then ethanol precipitated. (The EcoRI site in pBK53wt is further away from the T3 priming site than SalI.)

Exonuclease III reactions contained 30μg doubly-digested plasmid, 1X Exo III buffer (Stratagene), 10mM freshly diluted β-mercaptoethanol and 250 units exonuclease III in a final volume of 300μl. The exonuclease III was added last and the reaction was incubated at ambient temperature (22°C). At 1 minute intervals following the addition of the exonuclease, 12.5μl aliquots were taken and mixed with diluted 10X Mung Bean Nuclease Buffer (Stratagene) so that the DNA was diluted in a final volume of 100μl 1X Mung Bean Nuclease Buffer. These aliquots were snap-frozen in a dry-ice/methanol bath until all aliquots had been taken. The exonuclease was inactivated by heating the tubes to 68°C for 15 minutes and then placed on ice. The DNA in each aliquot was blunt-ended with 15 units mung bean nuclease (Stratagene; diluted in 1X Mung Bean Dilution Buffer) at 30°C for 30 minutes. 500ng of each aliquot was digested with BglII and run on a 1% agarose gel to check the extent of the deletions.

6.5μl of each of the nuclease-treated DNA was recircularised in separate 20μl ligation reactions containing 3 units T4 DNA ligase and 1X Ligase Buffer (Promega) at 4°C overnight and then transformed into XL1BlueMRF' cells. Individual colonies from each of the samples were mini-prepped and analysed on a 1% agarose following double-digestion with SacI and BglII. 12 clones were identified as containing nested deletions of the p53wt insert and were sequenced using the T3 primer.

2.5.18 Site-directed Mutagenesis

Double-stranded, site-elimination site directed mutagenesis (Fig. 2.8) was used to introduce corrective mutations or other sequence modifications into plasmids. In this procedure double-stranded DNA is denatured and one or more 5'-phosphorylated mutagenic primers are annealed to one strand of the plasmid. Both primers are extended with a mixture of T7 DNA polymerase and T4 DNA ligase to complete the mutagenic strand. At least one of the primers mutagenises a non-essential, unique restriction site in the plasmid so that it eliminates restriction by that enzyme in plasmids derived from the mutagenic strand (Selection Primer). The other primer introduces a specific mutation in the region of interest in the plasmid (Mutagenic Primer). One primer suffices in the case that elimination of a restriction site and introduction of a specific mutation can be accommodated in one oligonucleotide. All the plasmid DNA in the mutagenesis reaction is incubated with enzyme that corresponds to the non-essential, unique restriction enzyme present in the unmutated strand in order to eliminate plasmids that have not incorporated the Selection Primer. Plasmids containing mutagenic strands remain undigested and are transformed more efficiently into XL*mutS* competent cells. In XL*mutS* cells selection of the strand which is chosen to repair the mismatches between the mutagenic and parental strands is random. This means that 50% of transformants will contain mutant plasmids. The transformants are grown in liquid culture overnight and mini-prepped. The mini-prep DNA is subjected to a further restriction digestion with the selection enzyme and transformed into *E. coli* wild-type for the *mutS* gene (i.e. any of the usual laboratory strains). Since supercoiled DNA transforms *E. coli* much more efficiently than linearised DNA, individual transformants from the secondary transformation will most likely contain mutant plasmids and ~70-95 % will contain the desired mutation.

250pmol of each of the oligodeoxyribonucleotide primers N29 (10734: 5'-GCTGTGACTATGACACAAGTCTCCCTTTC-3'), N30 (10735: 5'-GCCTATCCACAGATGCTACGGAACACTCCT-3'), NEO (12210: 5'-GCCAACGCTATGTCCTGATAGGGGTCCCCCACACCCAGCCGGCC-3') and

W26 (12482: 5'-ACCAGCCTGACATGATCCGAAATCAC-3') were 5'-phosphorylated with T4 polynucleotide kinase (PNK) (Promega) in a 100µl reaction containing 2mM ATP and 1X PNK Buffer at 37°C for 1 hour.

Mutagenesis was carried out using reagents provided in the Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene). 25pmol of each of the primers N29, N30 (Mutagenic) and NEO (Selection) were mixed with 0.25pmol supercoiled pOPRNedd2 plasmid DNA in a 20µl reaction containing 1X Mutagenesis Buffer (Appendix A). Similarly, 25pmol of primer W26 (Mutagenic and Selection) was added to 0.25pmol of pGEM-WAF1. The DNA was denatured by boiling for 5 minutes, placed on ice for 5 minutes and the primers were annealed to the DNA by placing the tubes at ambient temperature for 30 minutes. The primers were extended and mutagenic strands ligated using a mixture of T7 DNA polymerase and T4 DNA ligase in the presence of 1mM dNTPs and 1X Mutagenesis Buffer for 1 hour. The polymerase and ligase were inactivated by heating the tubes to 75°C for 15 minutes.

7µl Buffer #7 and 20 units CspI were added to the pOPRNedd2 mutagenesis reaction and the volume adjusted to 70µl with DDW. 20 units KpnI and 29µl DDW were added to the pGEM-WAF1 mutagenesis reaction so that the final buffer conditions were 0.5X Mutagenesis Buffer. The restriction enzyme reactions were incubated at 37°C for 1 hour and 1/10 volume of the restriction digests were transformed into 90µl XL*mutS* competent *E. coli* that had been pre-treated with 25mM β-mercaptoethanol on ice for 10 minutes. The cells were incubated with the DNA in Falcon 2059 tubes on ice for 30 minutes, heat-shocked at 42°C for 45 seconds and placed on ice for 2 minutes before the addition of 0.9 ml SOC medium. The bacteria were then incubated at 37°C for 1 hour in an orbital incubator at 220 rpm. 3 ml of 2X YT broth (Appendix A) containing 100µg/ml amplicillin was added and the cultures were grown overnight at 37°C in the orbital incubator. The overnight XL*mutS* cultures were mini-prepped and mutant plasmids were enriched for by restriction with the appropriate enzyme (CspI or KpnI) in a 20µl reaction. 1µl of each digest was then transformed into 20µl XL1Blue competent cells and individual colonies were mini-prepped and screened for the desired mutation(s) by sequencing.

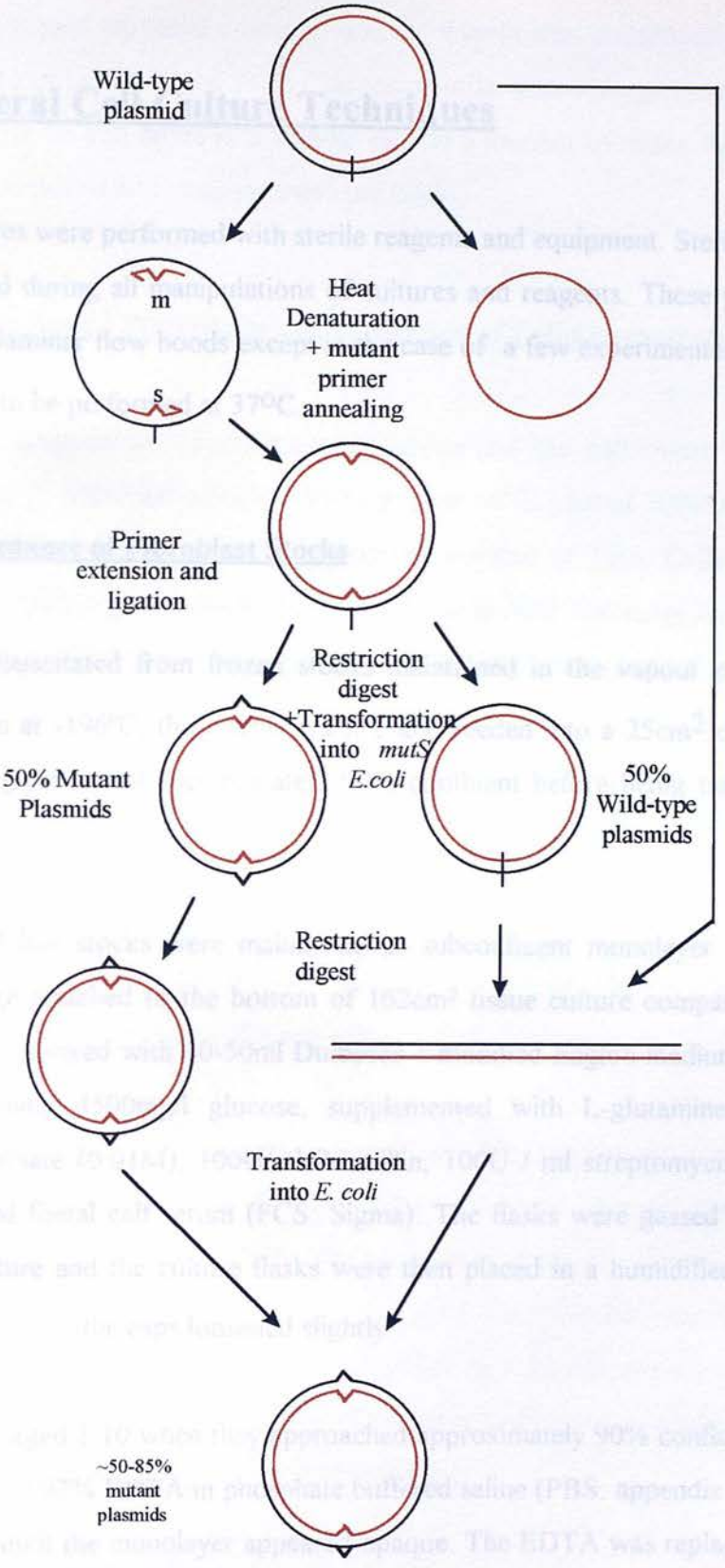


Fig. 2.8. Site elimination, site-directed mutagenesis. m: mutant primer; s: selection primer; r: restriction enzyme site.

2.6 General Cell Culture Techniques

All cell cultures were performed with sterile reagents and equipment. Sterile technique was employed during all manipulations of cultures and reagents. These were carried out in sterile laminar flow hoods except in the case of a few experimental procedures that required to be performed at 37°C.

2.6.1 Maintenance of Fibroblast Stocks

Cells were resuscitated from frozen stocks maintained in the vapour phase above liquid nitrogen at -196°C, thoroughly washed and seeded into a 25cm^2 culture flask (Costar) and grown until approximately 90% confluent before being transferred to larger flasks.

Fibroblast cell line stocks were maintained as subconfluent monolayer cultures by regular passage attached to the bottom of 162cm^2 tissue culture compatible plastic flasks (Costar) covered with 40-50ml Dulbecco's modified Eagle's medium (DMEM: Sigma) containing 4500mg/l glucose, supplemented with L-glutamine (0.1mM), sodium bicarbonate (0.01M), 100U/ml Penicillin, 100U / ml streptomycin and 10% heat-inactivated foetal calf serum (FCS: Sigma). The flasks were gassed with a 5% CO_2 / air mixture and the culture flasks were then placed in a humidified 5% CO_2, 37°C incubator with the caps loosened slightly.

Cells were passaged 1:10 when they approached approximately 90% confluence: cells were washed in 0.02% EDTA in phosphate buffered saline (PBS: appendix A) for 20-30 seconds or until the monolayer appeared opaque. The EDTA was replaced by 4ml of a solution of 0.1% trypsin in PBS and the monolayer was detached and disaggregated by gentle agitation. The resulting cellular suspension was transferred to

a conical-bottomed universal container and the trypsin was inactivated by adding an equal volume of DMEM/10% FCS. Clumps of cells were disaggregated by drawing the suspension up and down in a Pasteur pipette a number of times. Approximately 0.1 volume of this (0.8ml) was reseeded per flask.

2.6.2 Harvesting of Fibroblasts and Estimation of Cell Concentration

Cells were harvested by trypsinisation as above and the cells were collected by centrifugation in universal tubes at 1000rpm in an MSE Mistral 3000 centrifuge for 10 minutes and resuspended in an appropriate volume of PBS. Cells were either repelleted by centrifugation for storage at -20°C or seeded into fresh flasks or plates after estimation of cell concentration using a Neubauer improved haemocytometer.

2.7 Transfection of Plasmid DNA into Fibroblast Cell Lines

2.7.1 Plating of Cells Prior to Transfection

Cells were harvested by trypsinisation and placed into a universal tube. The cells were then passed once through a 19G needle to disperse clumps and diluted in complete medium (DMEM/10%FCS). The concentration of cells was then estimated using an improved Neubauer haemocytometer and 5-8 x 10^5 cells were diluted in 10 ml DMEM/10%FCS. This suspension was added to a 10 cm tissue culture plate, swirled to evenly distribute the cells over the surface and placed in a humidified, 5% CO_2, 37°C incubator for 24 hours before transfection.

2.7.2 Calcium Phosphate Precipitation (Chen and Okayama, 1987)

In this protocol (Chen and Okayama, 1987) which is a modification of the original HEPES method (Wigler *et al*, 1977; Graham and Van der Eb, 1973), DNA is mixed with a calcium chloride and a slightly acidic (pH6.95-6.98) buffered solution of sodium phosphate and this is added onto cells and incubated overnight at 35°C with 3% CO_2. As the pH of medium slowly rises during the incubation, a calcium phosphate / DNA precipitate forms and settles onto the cells. Some of this precipitate is then taken up by the cells.

20-35µg supercoiled Qiagen-column-purified plasmid DNA was mixed with 500µl 0.25M $CaCl_2$ (Sigma). 500µl 2X BBS pH6.95 (Appendix A) was mixed with the DNA solution and incubated at ambient temperature for 10-20 minutes. The DNA / $CaCl_2$ / BBS mixture was then added dropwise to a plate of cells whilst swirling the medium. The plate was then incubated for 16-24 hours in a humidified 3% CO_2, 35°C incubator. Then plates were then washed twice with 5 ml PBS and 10 ml of DMEM / 10%FCS was added and the plates returned to a 37°C, 5% CO_2 incubator. Transient transfections were incubated for 48-72 hours following addition of the DNA.

2.7.3 Selection and Cloning of Stable Transfectants

For stable transfections, washed transfected cells were incubated overnight before being split 1:10 by trypsinisation into fresh plates and incubated overnight. The medium was then aspirated and replaced with medium containing the appropriate antibiotic (200µg/ml hygromycin B (Boehringer Mannheim), 300µ/ml active G418 (Geneticin: Life Technologies) or 1µg/ml puromycin (Clontech)). Selective medium was replaced every 2 days for 10-14 days. Plates were then washed with PBS and clones were picked using a 200µl pipette tip into wells of 96-well plates (Costar) containing 100µl 0.1% trypsin in PBS. Cells were then fed with 150µl of selective medium and replaced in an incubator. When clones reached near-confluence they were

transferred to wells of a 24 well plate (Nunc) and subsequently into 6 well plates and then 75 cm^2 flasks (Costar).

2.7.4 Colony formation assay.

Cells were transfected and divided into replicate plates in selection medium for 11-14 days. Cells were then rinsed twice in PBS, fixed in 70% ethanol for 5 minutes and stained with 20% Giemsa in PBS for 10 minutes. Plates were rinsed with tap water and air dried. The number of colonies per plate was counted.

2.7.5 Histochemical stain for β-galactosidase (Lim and Chae, 1989).

Cells transiently transfected with plasmid pCMV-β were washed twice in PBS and fixed in 0.0% glutaraldehyde for 10 minutes. Plates were washed four times with PBS and stained at 37°C with X-gal solution: 0.2% X-gal (from a 2% stock in dimethylformamide) in 1mM $MgCl_2$, 150mM NaCl, 3.3mM $K4Fe(CN)_6.3H_20$, 3.3mM $K_3Fe(CN)_6$, 60mM Na_2HPO_4, 40mM NaH_2PO_4. Several fields of cells were counted and transfection efficiency was expressed as the percentage of cells that stained blue for β-galactosidase.

2.8 Northern Blot Analysis

2.8.1 Formaldehyde Gel Electrophoresis and Northern Transfer

Total RNA samples (10μg) were mixed with 3 volumes of RNA Sample Buffer (Appendix A) and heated to 55°C for 15 minutes. 0.2 volume of RNA Loading Buffer (Appendix A) was added and the samples were loaded into the wells of a 100ml, 1% denaturing agarose gel (1% agarose, 1 X MOPS Running Buffer (Appendix A), 18%

formaldehyde in DEPC/DDW) poured in a DEPC-treated Anachem Origo H3 gel tray. The gel was submerged in 1X MOPS Running Buffer and electrophoresed at 75mA until the bromophenol blue reached 70-75% of the length of the gel.

The RNA in the gel was then transferred to a positively-charged nylon membrane (Zetaprobe GT: BioRad) using a VacuGene XL vacuum blotter (Pharmacia). A piece of nylon membrane cut to the size of the gel was placed upon the porous support in the blotter and then surrounded with a plastic mask cut so that it overlapped the membrane by approximately 5mm on all sides. The gel was then placed on top of the membrane so that it overlapped the inside edges of the mask. The gel was covered in DEPC/DDW and a vacuum of 50-55mBar was applied to the blotter for no more than 5 minutes. The excess water was removed and promptly replaced with DEPC-treated and autoclaved 20X SSC so that the level of SSC was 2-3 times the thickness of the gel. The gel was blotted (still under vacuum) for 30 minutes after which the blotter was disassembled and the filter washed in 20X SSC for no more than 5 minutes before being dried for 30 minutes between two pieces of 3MM paper. The RNA was fixed to the membrane by baking *in vacuo* at 80°C for 30 minutes in a gel drier. Fixed filters were stored dry between two pieces of 3MM paper in plastic bags at 4°C if required.

2.8.2 DNA/RNA Hybridisation

Filters were prehybridised overnight at 42°C in hybridisation bottles containing 15ml of a prehybridisation solution containing 5X SSPE (Appendix A), 5X Denhardt's solution (Appendix A), 100μg/ml denatured, sonicated calf thymus DNA (Gibco) and 50% deionised formamide (Appendix A). The following day a double-stranded DNA probe was labelled and purified as detailed in the section on DNA/DNA hybridisation and boiled before being added to 15ml of hybridisation solution (prehybridisation solution plus 5% dextran sulphate) at 42°C. The prehybridisation solution was discarded and replaced with the hybridisation/probe mixture. Hybridisation was continued for 24 hours at 42°C.

Probed blots were washed twice for 15 minutes at 20°C in 1X SSC, 0.1% SDS and twice for 15 minutes at 20°C in 0.25X SSC, 0.1% SDS. The filters were then sealed moist in plastic bags and exposed to Fuji R-X film at -70°C in an autoradiography cassette with intensifying screens for at least 2 days.

2.9 Indirect Immunofluorescence for LacI

5×10^4 cells from hygromycin B-resistant clones obtained from transfections with p3'SS were plated into wells of 8 well Lab-Tek chamber slides (Nunc). One well per slide was seeded with untransfected Rat1 cells as a negative control. The plated cells were incubated in 300µl DMEM/10%FCS at 37°C, 5%CO_2 overnight. The medium, chambers and rubber gaskets were removed and the slides were washed in PBS three times before fixing the cells *in situ* in 4% formaldehyde in PBS for 10 minutes. The cells were then permeablised in 0.1% Tween-20 in PBS for 5 minutes and washed in 5 changes of PBS. The washed cells were incubated with rabbit polyclonal anti-*lacI* serum (Stratagene) at a dilution of 1:100 in PBS, 5% normal porcine serum (Dako) for 1 hour. The slides were then washed 5 times in 0.1% Tween-20, 0.5% bovine serum albumen fraction V (BSA: Sigma) in PBS. The slides were then incubated with fluoresceine isothiocyanate (FITC)-conjugated porcine anti-rabbit Ig serum at 25µg/ml in 0.1% Tween-20, 0.5% BSA in PBS for 1 hour. After washing 5 times in 0.1% Tween-20 in PBS the slides were mounted in an aqueous mountant and coverslipped. The fluorescence was viewed using a fluorescence microscope fitted with an FITC filter set.

Chapter 3.

3. Apoptosis Induced by p53 is Dependent upon the Cellular Context: Use of a Temperature-Sensitive p53.

3.1 Introduction.

Many tumours are resistant to chemotherapy, either intrinsically or following an initial partial response. A number of pharmacokinetic explanations may account for this, including over-expression of the multidrug resistance gene *mdr*1, over-expression of drug detoxication enzymes, or alteration of the drug target, for example topoisomerase II isoform. However despite intensive study of drug-target interactions, and drug metabolism, it is clear that in many instances drug resistance is associated with a failure of induction of apoptosis, even after an appropriate triggering event. Since many anti-cancer drugs and ionising radiation damage DNA, the response of the cell in recognising injury and proceeding to repair or apoptosis is of paramount importance (Hickman, 1992).

Entry to apoptosis is regulated by a number of genes (see Chapter 1, General Introduction: Apoptosis), each of which may show abnormal expression or function in cancer. In Rat-1 fibroblasts cell cycle arrest or serum deprivation in the presence of constitutive expression of the c-*myc* oncogene can cause apoptosis (Evan *et al,* 1992). By contrast, over-expression of *bcl*2 directly inhibits apoptosis in both normal and neoplastic cells (Veis *et al,* 1993; Sentman *et al,* 1991; Miyashita and Reed, 1993; Miyashita and Reed, 1992; Hockenberry *et al,* 1990) and prevents c-*myc* driven

apoptosis (Wagner *et al*, 1993). More recently evidence has accumulated implicating the tumour suppressor gene p53 in an injury-response pathway leading to apoptosis. Thymocytes and myeloid progenitor cells from p53 knockout mice, fail to undergo *induced* apoptosis in the absence of a wild-type p53 allele following etoposide or ionising radiation treatment but not apoptosis associated with ageing *in vitro* or nonclastogenic insults such as dexamethasone treatment. (Lotem and Sachs, 1993; Lowe *et al*, 1993; Clarke *et al*, 1993). Furthermore over-expression of wild-type p53 in a variety of cancer-derived cell lines such as M1 myeloid leukaemia (Yonish-Rouach *et al*, 1991), murine erythroleukaemia (Ryan *et al*, 1993) and HT29 colon carcinoma (Shaw *et al*, 1992) resulted in an increase in *spontaneous* apoptosis.

By contrast, studies of p53 null fibroblasts grown in primary culture have failed to detect alteration in cell survival characteristics after DNA damage as compared to normal primary fibroblasts (Slichenmeyer *et al*, 1993). In the latter experiments, cells were isogenic apart from p53 status. This suggests that other factors, including cell lineage and expression of oncogenes may modulate the effects of p53 on cellular physiology. In both experimental and human tumorigenesis p53 inactivation is believed to be a late event and is therefore superimposed on a series of progressive genetic abnormalities, such as activation of *ras* oncogenes (Fearon and Vogelstein, 1990).

The aim of this part of the project was to study the role of p53 in apoptosis of rodent fibroblasts in culture. In particular, two aspects were investigated: 1) to investigate the effects of DNA damage upon cells in which p53 phenotype can be independently modulated and 2) the interaction between p53 and apoptosis in the context of c-*myc* overexpression.

Firstly, cells expressing p53val135 were utilised in order to investigate the effect of wild-type and mutant p53 phenotype both upon cell cycle activity and upon the apoptotic response to DNA damage induced by two chemotherapeutic drugs known to cause DNA strand breaks and apoptosis, namely etoposide (VP16) and bleomycin.

Secondly, transfection experiments were designed such that expression of p53 and c-*myc* activity could be exogenously regulated. The temperature-sensitive mutant p53, p53val135, was used which is capable of both wt and mutant properties. Also, activity of a c-*myc*-oestrogen receptor chimaera (*myc*-ER; (Eilers *et al*, 1989)) can be regulable by the addition of β-oestradiol to the medium. In the first instance, an attempt was made to isolate sublines of cells containing constitutive c-*myc* and p53val135. Further, transfection of cells expressing p53val135 (Clone 6) with a *myc*ER construct was attempted, with the intention of producing cells in which the activity of both genes could be regulated independently.

3.2 Materials and Methods.

Details of general materials and methods used, including basic cell culture technique and plasmid DNA preparation and manipulation are to be found in Chapter 2. The specific details of materials and experimental procedures used for the work forming the basis of this chapter are given below.

3.2.1 Eukaryotic Expression Vectors

Five eukaryotic expression vectors, containing either a mutant p53 gene (pLTRp53cGval135, pLTRp53cGphe132 and pLTRp53cGXK), the hygromycin B resistance gene (pHMR272) or a chimaera of the human c-myc oncogene and a portion of the human oestrogen receptor gene (myc-ER)(pMV7-MER) were obtained as plasmid DNA samples and were transformed into competent *Escherichia coli*. Samples of ampicillin (or where appropriate, hygromycin B) resistant cultures were stored at -70°C (for details see below). A sixth plasmid (pMV7) was reconstructed from pMV7-MER by removal of the myc-ER coding fragment from the vector as described below. Plasmid details are summarised in Table 3.1. Plasmid maps are included in Appendix C.

3.2.1.1 Plasmids Containing Mutant p53 Sequences

Plasmid pLTRp53cGval135 (Eliyahu *et al*, 1985) is based on pBR322 (Bolivar *et al*, 1977) and contains the 3.7kb XhoI-SacII fragment from the murine p53val135 genomic clone Ch53-7 (Bienz *et al*, 1984) replacing the corresponding 0.95kb XhoI-SacII region of a murine p53 cDNA clone (Pinhashi and Oren, 1984; Zakut-Houri *et al*, 1983). The resulting hybrid contains eight of the ten introns found in genomic p53 and encodes a temperature-sensitive missense mutant p53 with a substitution from

alanine to valine at position 135. The hybrid is placed downstream from the 5' proviral Harvey murine sarcoma virus long terminal repeat and is flanked at the 3' end by sequences containing the early SV40 polyadenylation site.

Plasmid pLTRp53cGphe132 is similar to pLTRp53cGval135 and was derived by gapped-duplex site-directed mutagenesis (Kramer *et al*, 1984) from pLTRp53cGval135. The p53phe132 mutant has a corrected codon 135 (alanine) and is non-temperature-sensitive (Michalovitz *et al*, 1990).

Plasmid pLTRp53cGXK is identical to the above plasmids except that a 2.75kb XhoI-KpnI fragment has been deleted from the p53 coding sequences (Kaczmarek *et al*, 1986). This plasmid potentially encodes only the first 14 residues of p53 (M. Oren, personal communication).

3.2.1.2 ***Plasmid pHMR272***

pHMR272 (Bernard *et al*, 1985) is a plasmid/cosmid vector based on the Col E 1 origin of replication and contains an aminoglycoside antibiotic resistance gene (aph)(Hm^r) cloned from *Streptomyces hygroscopicus* which codes for the enzyme hygromycin B phosphotransferase (Kaster *et al*, 1983). This gene is used as a selectable marker in *E. coli* when under the control of the prokaryotic promoter *p*1 and can be expressed under the influence of the herpes simplex virus thymidine kinase (HSV*tk*) promoter in eukaryotic cells (Bernard *et al*, 1985). Selection of cells expressing Hm^r in either prokaryotes or eukaryotes is performed by adding appropriate concentrations of hygromycin B (200-500mg/ml) to the medium. Selection is independent of aminoglycoside resistance coded for by neomycin/G418 resistance genes and therefore hygromycin B resistance can be used for selection in serial or cotransfection experiments.

3.2.1.3 Plasmids pMV7 and pMV7-MER

The retroviral expression vector pMV7 (Kirschmeier *et al,* 1988) is based on a derivative of pBR322 which contains a polyomavirus origin of replication as well as retaining the pBR322 origin and ampicillin resistance genes (Dailey and Basilico, 1985; Luskey and Botcham, 1981) It also contains a 4.1kb XhoI fragment encoding a modified Moloney murine sarcoma virus (MoMSV). This MoMSV derivative was produced by removing 3.95kb of MoMSV genomic sequences (coding for the viral *gag* genes and large parts of v-*mos* and the *pol* gene) by digestion with PstI (Perkins *et al,*, 1983). It retains both 5' and 3' LTRs and regulatory elements for virion RNA encapsidation and reverse transcription. An HSV*tk*/neomycin phosphotransferase cassette which acts as a dominant selectable marker in eukaryotic cells has been inserted 0.3kb 5' of the 3' LTR. Two unique cloning sites (EcoR I and Hind III) are found 0.5-0.6kb downstream of the 5' LTR start of transcription.

pMV7-MER (Eilers *et al,* 1989) contains a 2.4kb EcoR I fragment inserted into the unique EcoR I site of pMV7. This fragment consists of a chimaera of cDNA sequences coding for exons II and III of the human c-*myc* proto-oncogene (Stone *et al,* 1987) fused to cDNA sequences representing the hormone binding domain of the human oestrogen receptor (Kumar *et al,* 1986).

Table 3.1. Summary of Plasmids

Plasmid	Gene Coded†	Eukaryotic Promoter(s)‡	Selectable Marker(s)§	Size	Reference
pLTRp53cGval 135	Ts p53	Ha-MSV LTR	Amp^r	9.8kb	Eliyahu (1985)
pLTRp53cGphe 132	m p53	Ha-MSV LTR	Amp^r	9.8kb	Michalovitz (1990)
pLTRp53cGXK	dl p53	Ha-MSV LTR	Amp^r	7.05kb	Kaczmarek (1986)
pHMR272	aph	HSV*tk*	Hm^r	4.1kb	Bernard (1985)
pMV7-MER	myc-ER Tn5-neo^r	Mo-MSV LTR HSV*tk*	Amp^r Neo^r	10.4kb	Eilers (1989)
pMV7¶	---------- Tn5-neo^r	MoMSV LTR HSV*tk*	Amp^r Neo^r	7.97kb	Kirschmeier (1988)

¶ Reconstucted in this study from pMV7-MER

† Ts p53 = (Temperature-sensitive) murine p53val153; m p53 = (non-temperature-sensitive) mutant murine p53phe132; dl p53 = p53 deletion (XhoI-KpnI) mutant; aph = aminoglycoside (hygromycin-B) phosphotransferase gene; Tn5-neo^r = neomcin resistance gene from bacterial transposon 5

‡LTR = Long Terminal Repeat; Ha-MSV = Harvey murine sarcoma virus; MoMSV = Moloney murine sarcoma virus; HSV*tk* = herpes simplex virus thymidine kinase promoter

§Amp^r = ampicillin resistance (prokaryotes); Hm^r = hygromycin-B resistance (prokaryotes and eukaryotes); Neo^r = neomycin resistance (eukaryotes)

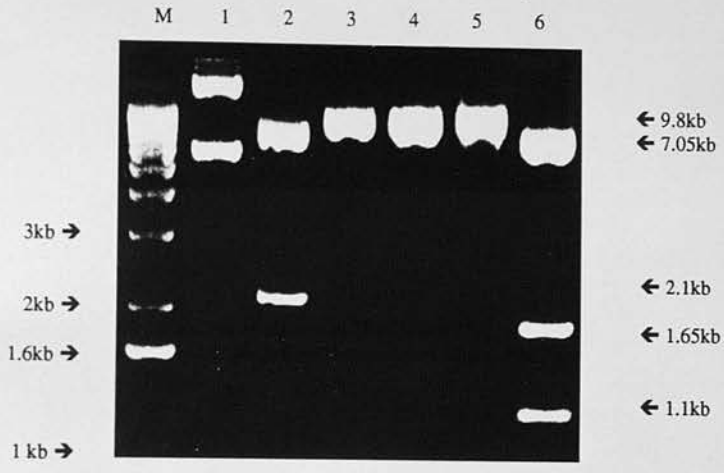


Fig. 3.1 : Restriction endonuclease analysis of plasmid pLTRp53cGval135.
M: kilobase marker; Lane 1: uncut; Lane 2: BamH I; Lane 3: Sal I; Lane 4: PvuI; Lane 5: XhoI; Lane 6: XhoI and KpnI.

Fig. 3.2 : Determination of linearising restriction sites for plasmid pLTRp53cGval135.
M: kilobase marker; Lane 1: NruI; Lane 2: NdeI; Lane 3: SphI; Lane 4: PvuI; Lane 5: AatI; Lane 6: EcoR V; Lane7: SalI.

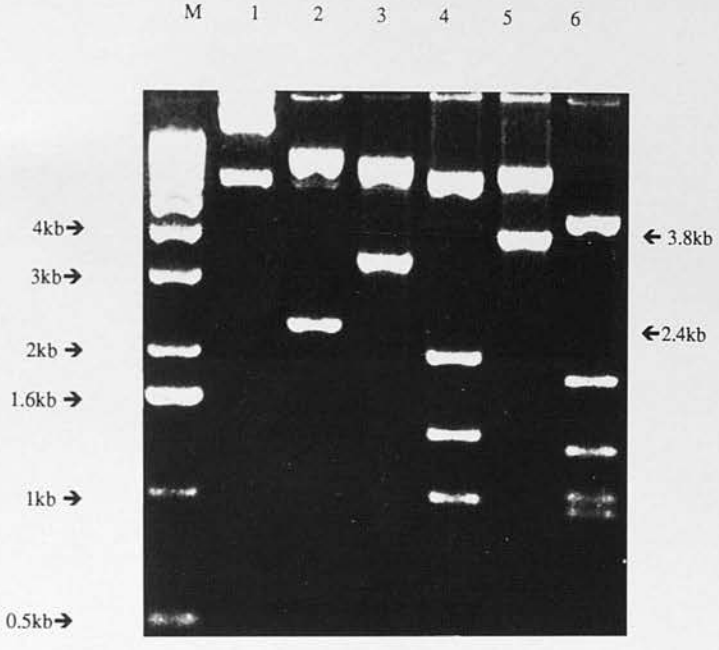


Fig. 3.3 : Restriction endonuclease analysis of plasmid pMV7-MER.
M: kilobase marker; Lane 1: uncut; Lane 2: EcoR I; Lane 3: BamH I; Lane 4: EcoR I and BamH I; Lane 5 Xho I; Lane 6: Pst I.

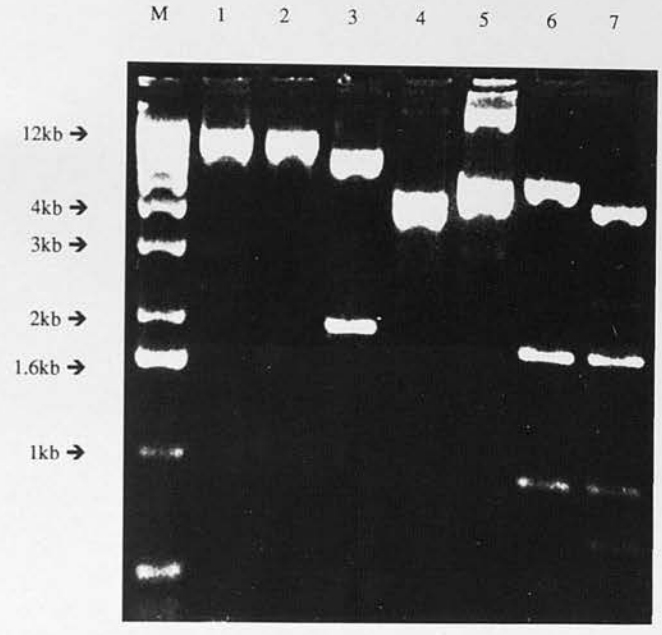


Fig. 3.4 : Restriction endonuclease analysis of plasmid pMV7.
M: kilobase marker; Lane 1: EcoR I; Lane 2: BamH I; Lane 3: EcoR I and BamH I; Lane 5 Xho I (note doublet of 3.8kb and 4.17kb bands); Lane 6: uncut; Lane 7: Pst I; Lane 8: Pst I and EcoR I.

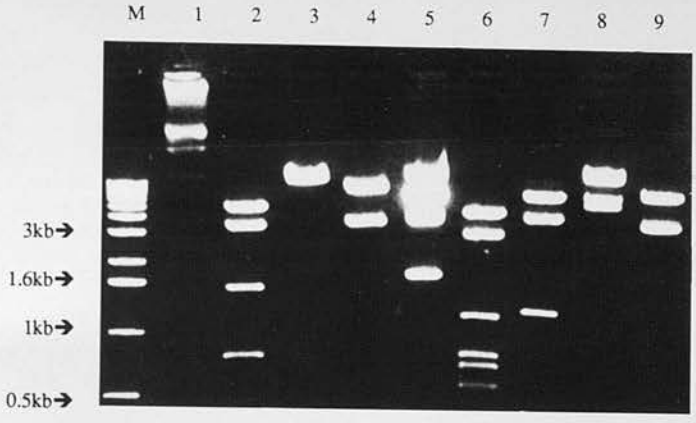


Fig. 3.5 : Determination of linearisation sites for plasmid pMV7-MER. No unique sites were found. However, Xho I cuts twice, bracketting the sequences essential for function of this expression vector.
M: kilobase marker; Lane 1: uncut; Lane 2: SmaI; Lane 3: XbaI ; Lane 4: SalI; Lane 5: AccI; Lane 6: PvuI; Lane 7: BglI; Lane 8: AatI; Lane 9: XhoI.

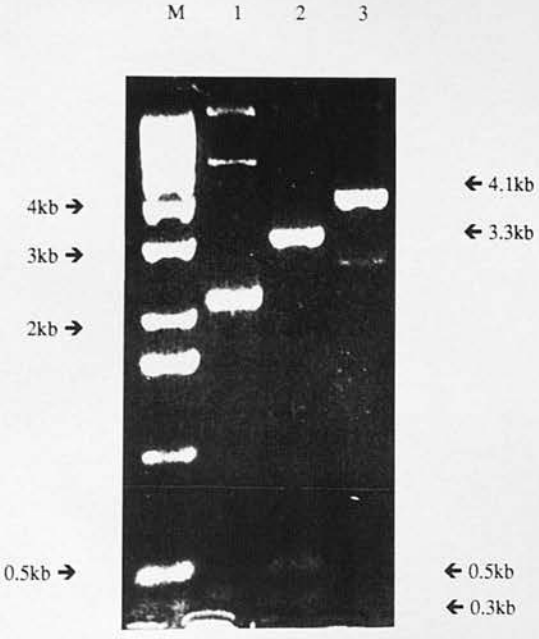


Fig. 3.6 : Restriction endonuclease analysis of plasmid pHMR272.
M: kilobase marker; Lane 1: uncut; Lane 2: EcoRI; Lane 3: HindIII.

3.2.2 Fibroblast Cell Lines

A number of rodent fibroblast cell lines were procured and maintained in culture in which expression of the cellular oncogene c-myc or the temperature sensitive p53 mutant p53val135 had been augmented by genetic manipulation. The cell lines were originally produced by insertion of the corresponding gene into high expression vectors and subsequent introduction into the eukaryotic cells by either infection with recombinant retroviruses (Brown and Scott, 1987) or transfection of cells by the calcium phosphate co-precipitation technique (Graham and Van der Eb, 1973). Details of cell lines are summarised in Table 3.2.

3.2.2.1 Clone 6 Cells and RcGphe132.4 cells.

This cell line (kindly provided by M. Oren) constitutively expresses the temperature sensitive p53 mutant, p53val135 which adopts wild type conformation and functional properties at permissive temperatures (32°C) and mutant conformation and properties at 37°C (Michalovitz *et al,* 1990). Clone 6 is a cell line derived from low passage rodent embryo fibroblasts transformed by murine p53val135 and a human mutationally-activated c-Harvey-*ras*1 gene (Michalovitz *et al,* 1990) using the calcium phosphate co-precipitation technique (Graham and Van der Eb, 1973). Clones were propagated from foci that developed as a result of co-operation between Ha-*ras* and p53val135 in transformation of the transfected rat embryo fibroblasts at 37°C (Eliyahu *et al,* 1984; Michalovitz *et al,* 1990). In Clone 6, p53val135 is under the transcriptional control of a Harvey murine sarcoma virus long terminal repeat (LTR) and is encoded by plasmid pLTRp53cGval135 (Kaczmarek *et al,* 1986; Eliyahu *et al,* 1985). Activated Ha-*ras* in these cells is encoded by plasmid pEJ6.6 (Shih and Weinberg, 1982). RcGphe132.4 cells contain a temperature-stable p53phe132 transgene in addition to activated c-Ha-*ras*1. The level of p53 expression in Clone 6 and RcGphe132.4 is similar (M. Oren, personal communication). All

manipulation of cell lines and counting was performed at the selected temperature to minimise the risk of inadvertent p53 conformational shifts.

3.2.2.2 Rat-1A/mycΔ145-262 and Rat-1A/mycΔ106-143 Cells

These rodent fibroblast cell lines were obtained from Dr. G. I. Evan (ICRF, London) and constitutively express either an active or an inactive deletion mutant of the human c-*myc* oncogene. Both of these lines were derived from the methylcholanthrene-treated Fischer rat embryo fibroblast cell line Rat1A (see Chapter 2: General Materials and Methods). In-frame deletion mutants of cDNA clones of exons II and III of the human c-*myc* oncogene (Stone *et al*, 1987) were previously inserted into the retroviral expression vector pMV6 and transfected into the packaging cell line Ψ2 (Stone *et al*, 1987; Mann and Mulligan, 1983). Helper-free ecotropic retoviral stocks were then harvested from the medium above transfected Ψ2 cells and were used to infect Rat1A cells according to the procedure outlined by Brown and Scott (1987) and stable incorporation of the vector was selected for on the basis of neomycin resistance (Penn *et al*, 1990; Stone *et al*, 1987).

The Rat-1A/*myc*Δ145-262 cell line is a pool of 150-250 neomycin resistant colonies derived from Rat-1A cells infected with retroviruses encoding the c-*myc*Δ145-262 deletion mutant. This mutant has been shown to be fully active in autosuppression of endogenous c-*myc* expression (Penn *et al*, 1990), co-operation with Ha-*ras* in transformation of Rat-1A fibroblasts (Stone *et al*, 1987) and is capable of inducing apoptosis in Rat-1A cells upon serum depletion (Evan *et al*, 1992). Rat1A/*myc*Δ106-143 cells express high levels of mRNA transcripts of the deletion mutant c-*myc*Δ106-143 but this mutant shows no autosuppression, cotransformation or apoptotic activity (Evan *et al*, 1992; Stone *et al*, 1987; Penn *et al*, 1990).

Table 3.2 Summary of Cell Lines

Cell Line	Parent Cells	Genes expressed†	Tx Method‡	Basis of selection§	References
Rat-1A/mycΔ145-262	Rat-1A	active c-myc Tn5-neo^r	retroviral infection	neo^r	Stone (1987) Penn (1990)
Rat-1A/mycΔ106-143	Rat-1A	crippled c-myc Tn5-neo^r	retroviral infection	neo^r	Stone (1987) Penn (1990)
Clone 6	REF¶	Ts p53 Ha-ras	$Ca_3(PO_4)$	focus formation	Pinhasi-Kimchi (1986) Michalovitz (1990)

¶ REF = early passage Fisher rat embro fibroblasts

† active c-myc = mycΔ145-262; crippled c-myc = mycΔ106-143; Tn5-neo^r = neomycin resistance gene from bacterial transposon 5

‡ Tx = transfection; $Ca_3(PO_4)$ = calcium phosphate coprecipitation metho

d

§ neo^r = neomycin (G418) resistance

3.2.3 Morphological Analysis of Cell Lines.

3.2.3.1 Phase Contrast Microscopy

Phase contrast microscopy was used to investigate the cellular morphology of fibroblasts adherent to the substratum and to examine the cultures for apoptotic cell death under the various experimental conditions described below. Monolayers of cells were examined at medium power (x100) and at high power (x320) using an inverted phase-contrast microscope (Leitz).

3.2.3.2 Transmission Electron Microscopy

Clone 6 cells were seeded in duplicate flasks and grown at 37°C for 24 hours. At this point one flask was moved to the 32°C incubator and cells in a duplicate flask were fixed *in situ* with 3% glutaraldehyde in 0.1M sodium cacodylate for 1 hour, and then washed and stored at 4°C in a 5% sucrose / sodium cacodylate pH 7.4 buffer for no longer than 48 hours. After 24 hours at 32°C cells were similarly fixed, washed and stored overnight. All cells were then post-fixed *in situ* with 1% osmium tetroxide in sodium cacodylate. Cells were then removed from the flasks by scraping and suspended in cacodylate buffer. A pellet containing cells from each sample was prepared by centrifugation. Pellets were dehydrated through graded alcohols to absolute ethanol, incubated twice in propylene oxide for 20 minutes and then impregnated with grades of araldite to 100% araldite. Samples were maintained at room temperature overnight in 100% araldite before polymerisation of the araldite at 56°C. Representative ultrathin (40-50nm) sections were cut on an ultramicrotome and stained with uranyl acetate and lead citrate. Specimen grids were examined using a transmission electron microscope (JEM-100: Jeol).

3.2.4 Evaluation and Quantitation of Apoptotic Cell Death

3.2.4.1 Morphological Evaluation of Apoptotic Cell Death

Apoptotic cells were recognised under the phase contrast microscope as rounded, highly refractile, spherical bodies. This assertion was confirmed by acridine orange fluorescence and by electron microscopy (data not shown). Attached apoptotic bodies were defined as those refractile bodies that appeared to be almost perfectly spherical and could not be induced to migrate across the field by gentle tapping of the microscope stage.

3.2.4.2 Quantitation of Cell Number and Apoptosis.

Reference points (3 per flask) were used to count directly the number of cells in x100 field using a 10 x 10 graticule. This permitted sequential counts at 20, 28 and 42h. after plating at 32°C or 37°C. Apoptotic cells adherent to the monolayer were counted at each time point as well as cell number. The apoptotic cells were recognised by virtue of their spherical, highly refractile appearance under phase contrast. These cells showed the classical appearances of apoptosis and were confirmed by electron microscopy and acridine orange fluorescence microscopy (Arends and Harrison, 1994).

Both the total number of cells per field and the total number of apoptotic bodies adherent to the monolayer were counted as described above using the phase contrast microscope. Clusters of very small apoptotic bodies were considered to have undergone fragmentation and were assumed to have derived from one cell. Spherical refractile bodies that occurred in pairs were suspected to be mitotic and, unless they were perfectly spherical and failed to exhibit anchoring pseudopodia, were ignored. The prevalence of apoptosis in the fields was expressed as the number of apoptotic (attached, non-mitotic, refractile) bodies as a percentage of the number of live cells per field ("percentage apoptosis").

3.2.5 Treatment of Cells with DNA Damaging Agents

Clone 6 cells were plated at a density of 2 x 10^4 cells/cm^2 and grown for 24 hours at 37°C. One group of cells was then removed to a 32°C incubator while treatment with drugs (dissolved in the appropriate vehicle) began with the cells at 37°C. Drug treatment of cells at 32°C was commenced 24 hours after the temperature shift. All procedures were carried out at the appropriate temperature with previously equilibrated reagents. It was assumed that washing effectively removed drug from the cells.

3.2.5.1 Etoposide

The dimethylepipodophyllotoxin, etoposide (VP-16), has been shown to cause double-stranded breaks in DNA by interfering with the breakage-reunion reaction of the enzyme topoisomerase II, resulting in stabilisation of enzyme-drug-DNA cleavable complexes (Liu, 1989). The level of cellular expression of topoisomerase II activity is dependant upon position in the cell cycle and determines the levels of drug-induced DNA cleavage and cell death (Long *et al*, 1986). However, the complexes and DNA breaks are reversible upon removal of the drug (Long *et al*, 1985) and there is dissociation between maximal topoisomerase II activity (G2/M) and maximal cytotoxicity (S phase)(Chow and Ross, 1987). It has been suggested that cells may be triggered to die by an interaction between the epipodophylloyoxin stabilised cleavable complexes and other cellular processes such as DNA and RNA synthesis or illegitimate recombination (Bae *et al*, 1988). Upon removal of etoposide, cells undergo a transient cell cycle arrest and and accumulate in G2 which correlates with inhibition of $p34^{cdc2}$ kinase activity (Lock and Ross, 1990b). Upon recovery of $p34^{cdc2}$ activity to abnormally high levels (after 12-24 hours in CHO cells), many cells enter mitosis and subsequently die by apoptosis after abnormal segregation of chromosomes (Lock and Ross, 1990a).

A stock solution of etoposide (VP-16; 100mM in DMSO)(Sigma) was diluted fresh for each experiment to 10mM in PBS. This suspension was added to the medium above the experimental cells to a final concentration of either 10μM or 50μM. After 1 hour, the medium was removed and the cells washed three times with PBS before adding fresh medium to each flask. Control cells were treated similarly with an equivalent volume of 10% DMSO in PBS.

3.2.5.2 Bleomycin.

Bleomycin sulphate is a mixture of complex glycopeptides produced by *Streptomyces verticillus*, bleomycinA2 and bleomycinB2 being the main constituents (Crooke and Bradner, 1976). Bleomycin has a portion that binds divalent metal ions such as Cu(II) and Fe(II) and a region that binds to DNA at specific sequences (eg, 5'-GC-3' and 5'-GT-3'; (Mirabelli *et al*, 1983; Mirabelli *et al*, 1982). Single and double strand breaks are made in DNA when bleomycin-Fe(II)-O_2 complexes bind to DNA and catalyse the reduction of O_2 to hydroxyl and superoxide radicals (Burger *et al*, 1986; Lown and Sim, 1977). Active or relaxed chromatin is more susceptible to bleomycin-induced strand breaks (Kuo, 1981). Bleomycin causes an accumulation of cells in G2 (with many cells exhibiting chromosomal abnormalities) (Watanabe *et al*, 1974; Hittleman and Rao, 1974; Nagatsu *et al*, 1972; Tobey, 1972) and apoptosis (Kuo and Hsu, 1978).

A stock solution of 1unit / ml bleomycin (Sigma) in PBS was prepared (1U = 1mg bleomycin A2) and added to the medium above experimental cells to a final concentration of 15μU/ml. After one hour cells were washed three times with PBS before adding fresh medium to the flask. Control cells were treated with an equivalent volume of PBS.

3.2.6 Cell Cycle Analysis

An EPICS CS (Electronically Programmable Individual Cell Sorter; Coulter Electronics) flow cytometer was used to provide a measure of the DNA content of Clone 6 cells under experimental conditions. Nuclei were isolated and stained with propidium iodide (Vindelov *et al*, 1983), and 1 x 10^4 cells were analysed on the flow cytometer. Histogram analysis was performed using the Easy 2 Software. No doublets were seen. Bromodeoxyuridine incorporation analysis was carried out using the Amersham Cell Proliferation Kit (cat. #: RPN20).

3.2.7 Stable Transfection of Plasmid DNA into Mammalian Cells

Two methods of the stable introduction of plasmid DNA into fibroblast cell lines were used. The calcium phosphate co-precipitation technique (Graham and van der Eb, 1973; Wigler *et al*,, 1977; Wigler *et al*, 1979) was used to co-transfect Rat1A-derived fibroblast cell lines with plasmids containing mutant p53 sequences and plasmid DNA containing the hygromycin B resistance gene (pHMR272). The electroporation method (Neumann *et al*, 1982) was used to introduce plasmids pMV7 and pMV7-MER, into Clone 6 cells.

Plasmids were linearised before transfection in order to maximise efficiency of incorporation of vector into recipient genomic DNA (Szostak *et al*, 1983; OrrWeaver *et al*, 1981).

Selection was performed with the appropriate antibiotics (G418 or hygromycin B; see Chapter 2 for details) added to the medium for a period of at least 1-2 weeks in order to select for cells with stably-incorporated vector sequences. In all transfection experiments, two samples of cells were similarly treated, one sample without the addition of DNA in order to confirm the efficiency of the selection conditions.

3.2.7.1 Linearisation and Ethanol Precipitation of Plasmid DNA

20-30μg of each plasmid was linearised by exhaustive digestion with the appropriate restriction enzyme. Candidate enzyme sites occurring only in non-essential portions of the vectors were shown to cut only in these regions by digestion of small samples of plasmid DNA and analysis of the resulting restriction fragments by agarose gel electrophoresis (Table 3.3 and Figs. 3.1 to 3.6).

A small sample of each digestion reaction was retained for electrophoresis in order to check that complete digestion had indeed occurred. Once digestion was confirmed, the DNA was precipitated with 2 volumes of absolute ethanol at 4°C for 30 minutes. The DNA was collected by microcentrifugation at 4°C for 20 minutes at 13000 rpm and briefly air-dried in a sterile laminar flow hood before use in transfection protocols.

3.2.7.2 Transfection: HEPES-based Calcium-Phosphate Coprecipitation Method

Graham and van der Eb (1973) described the optimal conditions for the uptake of coprecipitates of calcium phosphate (125mM) and DNA (5-30μg/ml) at pH 7.05 into adherent cells. It is thought that co-precipitated particles of appropriate size enter the cytoplasm by pinocytosis and may be transferred to the nucleus by an unknown mechanism. The calcium phosphate co-precipitation method can be used to establish stably transfected cell lines with multiple, integrated copies of vector DNA (Sambrook *et al*,, 1989).

Cells were harvested 24 hours before transfection, washed in PBS and drawn through a fine needle with a syringe to form a single cell suspension. Cells were plated at a density of 1 x 10^5 cells/cm^2 onto 100mm x 20mm tissue culture compatible Petri dishes (Costar) in 10ml GMEM/10% HINCS and placed in a humidified 5% CO_2 incubator at 37°C. Four hours before transfection the medium was changed and returned to the incubator. Linearised plasmid DNA (20-30μg of one p53-containing plasmid together with 2-3μg linearised pHMR272) was ethanol-precipitated (2.5.1) in a sterile microfuge tube, dried briefly in a sterile laminar flow hood and dissolved in

0.5ml 0.1 x TE (pH8.0) / calcium phosphate (250mM) solution. This solution was added drop-by-drop, with constant gentle mixing to 0.5ml 2 x HEPES buffered saline (2 x HBS: appendix A) in a sterile Falcon 2059 tube (Becton Dickinson). The mixture was then incubated at room temperature for 20-30 minutes to allow the DNA-calcium phosphate precipitate to form before addition of the entire mixture to the medium above the cells to be transfected. The dish was gently rocked to ensure complete mixing of the precipitate with the medium and the dish was returned to the incubator. After 16 hours the medium was aspirated, cells were washed with PBS and fresh medium applied. The cells were allowed a further 24 hours incubation before replating at 2 x 10^4 cells/cm^2 in selection medium.

3.2.7.3 Transfection: Electroporation Method

Electric field pulses applied across cellular suspensions are believed to facilitate DNA uptake into cells by direct entry into the cytoplasm and nucleus by formation of transient pores in cellular membranes. In this way single copies of cloned genes can be incorporated into eukaryotic cells (Boggs *et al*, 1986). On this basis, linearised plasmid vectors were transfected into fibroblasts by electroporation (Neumann *et al*,, 1982) using a Bio-Rad Gene Pulser and Capacitance Extender.

Plasmid DNA (20-30μg of each construct) was linearised by exhaustive restriction digestion with the appropriate enzyme, ethanol precipitated in a sterile microfuge tube and allowed to dry briefly in a sterile laminar flow hood before dissolving in 0.8ml sterile PBS. Approximately 10^7 fibroblasts grown to 70-80% confluence were harvested by trypsinisation, washed in PBS, centrifuged at 1000 rpm at 20°C for 5 minutes and then resuspended in the DNA / PBS solution. The resulting DNA / cell suspension was then transferred to a Gene Pulser cuvette (a chamber, two opposing sides of which are constructed from sheets of metal and act as parallel electrodes). Two discharges of an electric field of 0.23kV with a capacitance of 500μF (McMahon and Bradley, 1990) were applied across the DNA / cell suspension at ambient temperature. Cells were allowed to recover for 10 minutes before being drawn

through a fine needle with a syringe to form a single cell suspension (to help prevent co-operation between adjacent cells in selection) and seeded at an approximate density of 2×10^4 cells/cm^2 into 25cm^2 flasks or onto 10cm x 20mm tissue culture compatible Petri dishes (Costar) with an appropriate volume of GMEM/10% HINCS. Cells were selected and cloned as described in the General Materials and Methods section (Chapter 2).

Table 3.3. Restriction Analysis of Plasmids

Plasmid	Size	Diagnostic Enzyme(s)	Predicted Fragment Sizes	Linearisation Enzyme
pLTRp53cGval135 and pLTRp53cGphe132	9.8kb	EcoR I BamH I XhoI and KpnI	9.8kb 2.1kb, 7.7kb 1.1kb, 1.65kb, 7.05kb	Sal I
pLTRp53cGXK	7.05kb	EcoRI XhoI and KpnI	7.05kb 7.05kb	SalI
pHMR272	4.1kb	HindIII EcoR I	4.1kb 0.3kb, 0.5kb, 3.3kb	Hind III
pMV7-MER	10.4kb	EcoR I XhoI EcoR I and BamH I	2.4kb, 7.97kb 3.8kb, 6.6kb 1.0kb, 1.4kb, 2.0kb, 5.97kb	XhoI¶
pMV7	7.97kb	EcoR I XhoI EcoR I and BamH I	7.97kb 3.8kb, 4.17kb 2.0kb, 5.97kb	XhoI¶

¶ XhoI cuts twice in pMV7 and releases a 3.8kb fragment containing pBR322 derived sequences. The larger fragment contains retroviral sequences, HSV*tk*-neo^r, and the unique EcoR I cloning site into which the myc-ER construct has been inserted in pMV7-MER

3.3 Results

3.3.1 Cellular Morphology

3.3.1.1 Phase Contrast Microscopy

The majority of Clone 6 cells grown at 37°C in plastic culture flasks appeared as elongated spindle-shaped, slightly refractile cells with visible nuclei and multiple (3-5) nucleoli. A minority of cells at 37°C appeared more broadened, irregularly shaped and were less refractile (Fig. 3.7a). Nuclei were, in general, round or oval shaped, but some cells exhibited slightly irregular outlines. Rare, atypically large cells appeared to contain more than one nucleus per cell or contained one nucleus that was noticeably larger than cells of usual appearance. Mitoses were visible at a rate of about 3-5% in subconfluent cultures. When grown to confluence, the cells tended to overlap each other and eventually formed into foci (Fig. 3.10)

Most Clone 6 cells incubated at 32°C for a least 24 hours were larger, broader and much less refractile than at 37°C (Fig. 3.7b). These cells exhibited an abundance of criss-crossing, filamentous densities strewn about the cytoplasm. Mitotic cells were extremely rare after 24 hours incubation at 32°C. Cells kept at this temperature did not increase in number and did not become confluent. Few apoptotic cells were seen at either temperature in the monolayer or supernatant.

In contrast, Rat-1A/*myc*Δ145-262 and Rat-1A/*myc*Δ106-143 cells showed no change in morphology and only a small reduction in growth rate when placed at 32°C. There were an abundance of spherical, highly refractile cellular bodies, both attached to the monolayer and suspended in the medium above the Rat-1A/*myc*Δ145-262 cells (3.13b). These bodies were demonstrated to be apoptotic by the observation of nuclear fragmentation in samples of these bodies when stained with acridine orange

thus shows a moderate rate of apoptosis, but its induction is not temperature sensitive.

3.3.1.2 Transmission Electron Microscopy

Samples of Clone 6 cells previously incubated at either 37°C or 32°C were fixed *in situ* and processed for electron microscopy. Nuclei usually possessed one large, dense nucleolus, which sometimes contained some unusual, highly electron-dense inclusions. It was considered that there may have been less endoplasmic reticulum and less prominent nucleoli in cells incubated at 32°C than in cells incubated at 37°C. Otherwise, there were no obvious ultrastructural differences at the two temperatures (Fig 3.9).

3.3.2 Growth Properties and Serum Dependence

Cells from all three lines were seeded at equal density, grown in wells of a 24-well tissue culture plate for 24 hours (37°C, GMEM / 10% HINCS) and subsequently incubated in either a 37°C or a 32°C incubator in GMEM supplemented with 10%, 0.01% or 0% HINCS. The degree of viability of each cell line was estimated qualitatively in order to determine whether apoptosis could be induced in the cell lines under conditions of serum deprivation. Cells grown in the presence of 10% serum had the typical morphology and growth characteristics of cells grown as monolayers in the stock flasks. All the cell lines maintained their basal apoptotic rate and proceeded to confluence within 2-4 days.

As previously observed by Evan et al (1992), Rat-1A/*myc*Δ145-262 cells showed an increase in apoptotic rate when placed in reduced serum conditions. Rat-1A/*myc*Δ106-143 cells did not show this effect. In addition both cell lines maintained these characteristics when the experiment was repeated at 32°C

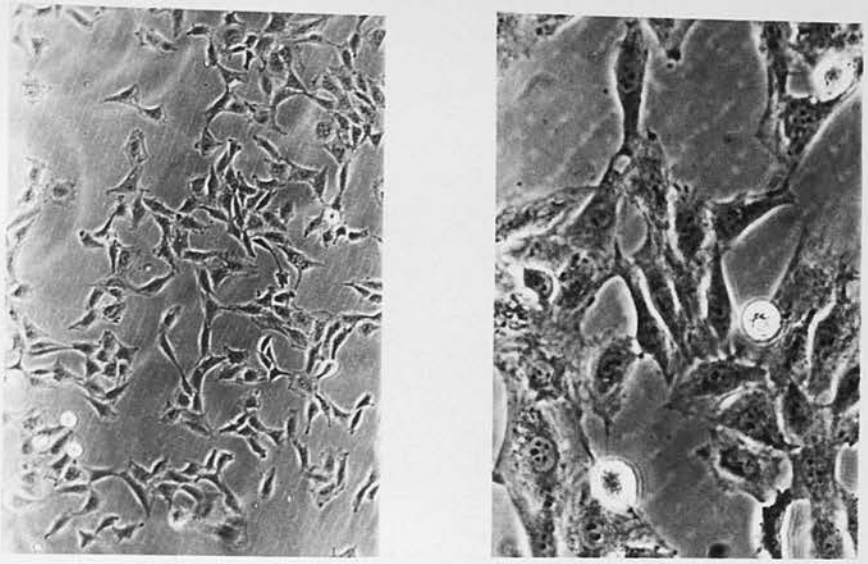


Fig. 3.7a : Clone 6 cells grown at 37°C. Cells are elongated, slightly refactile and possess prominent nuclei.
Phase contrast: Left: x 100; Right: x 320.

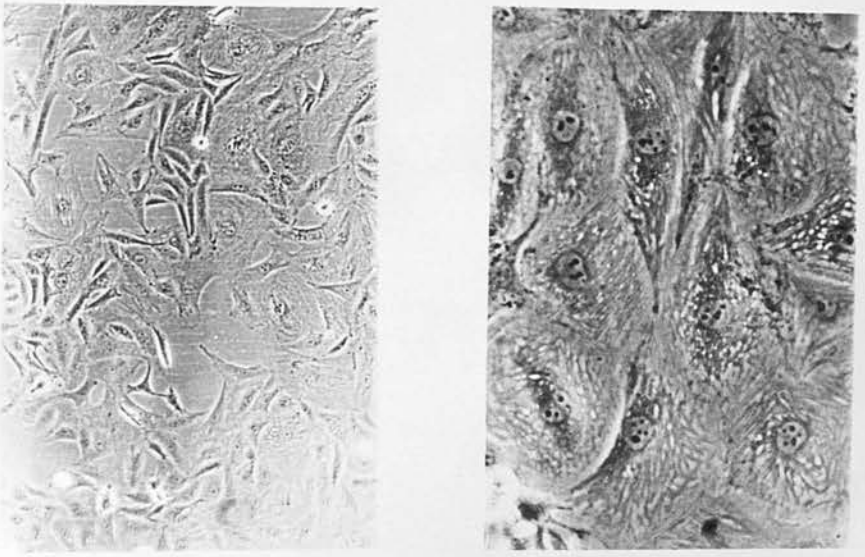


Fig. 3.7b : Clone 6 cells incubated at 32°C. Cells are spread out over the substratum and contain criss-crossing filamentous densities in the cytoplasm.
Phase contrast: Left: x 100; Right: x 320.

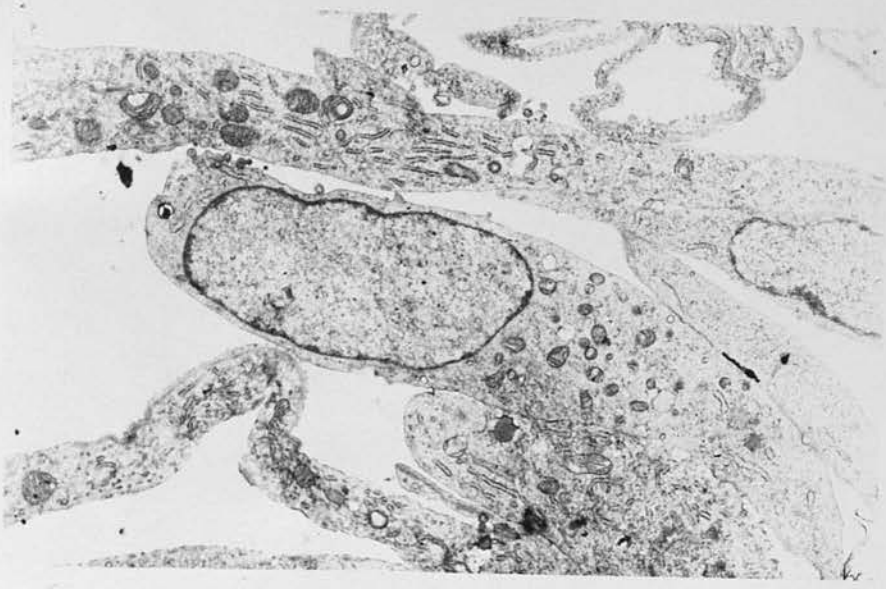


Fig. 3.9a : Clone 6 cells grown at 37°C.
TEM: x 4000

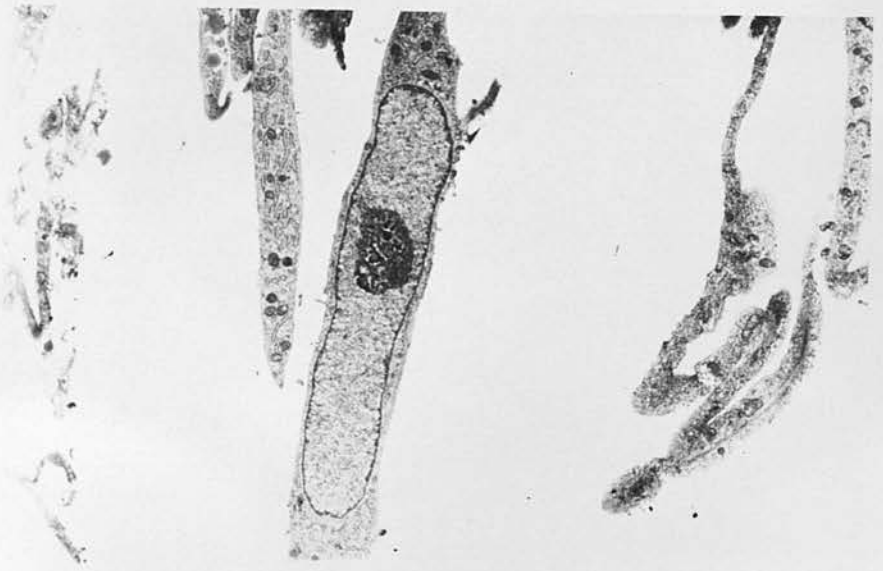


Fig. 3.9b : Clone 6 cells incubated at 32°C. Note electron dense inclusions in nucleolus.
TEM: x 3000.

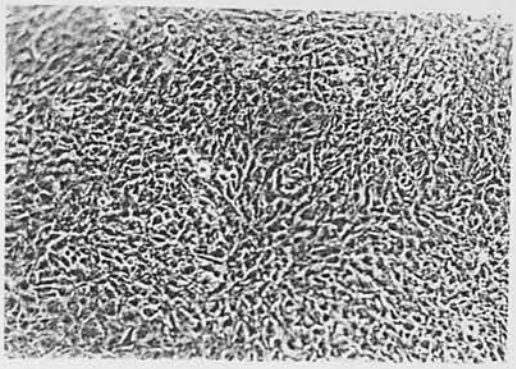


Fig. 3.10 : Clone 6 cells grown to confluence. Cells are highly refractile and grow on top of each other.
Phase contrast: Above: x 100; Below: x 320.

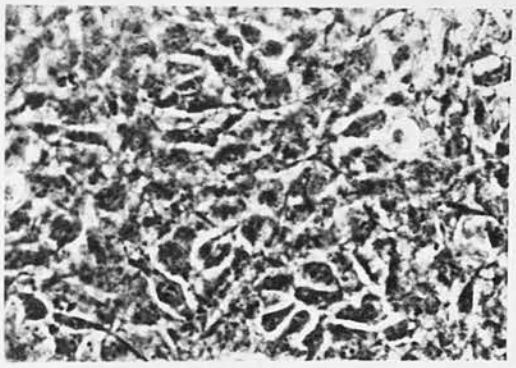


Fig. 3.11 : Clone 6 cells at 37°C 24 hours after treatment with 50μM etoposide. Note abundant apoptotic bodies.
Below: Phase contrast: x 320.

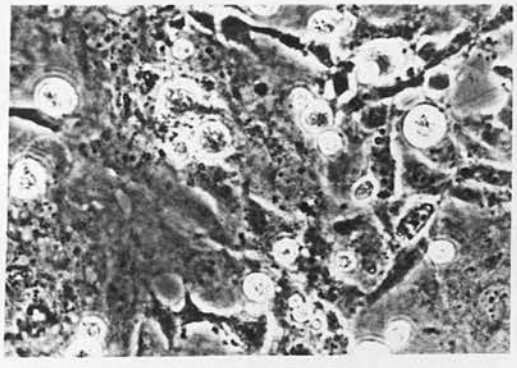


Fig. 3.12a : Rat1A/mycΔ106-143 at low density. Cells are elongated, refractile and slighlty overlapping. Observe the spherical, refractile properties of the apoptotic bodies.
Phase contrast: x 100.

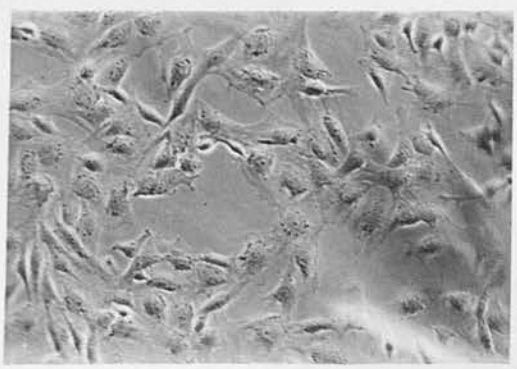


Fig. 3.12b : Clone 6 cells at low density. Note while some cells are elongated, others are broader and less refractile.
Phase contrast: x 100.

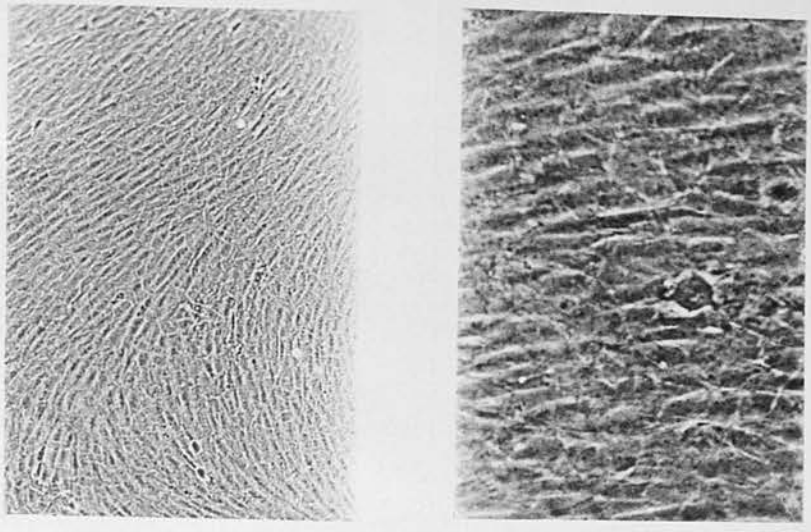


Fig. 3.13a : Rat1A/mycΔ106-143 cells grown to confluence. Cells are elongated, form fascicular arrays and do not overlap.
Phase contrast: Left: x 100; Right: x 320.

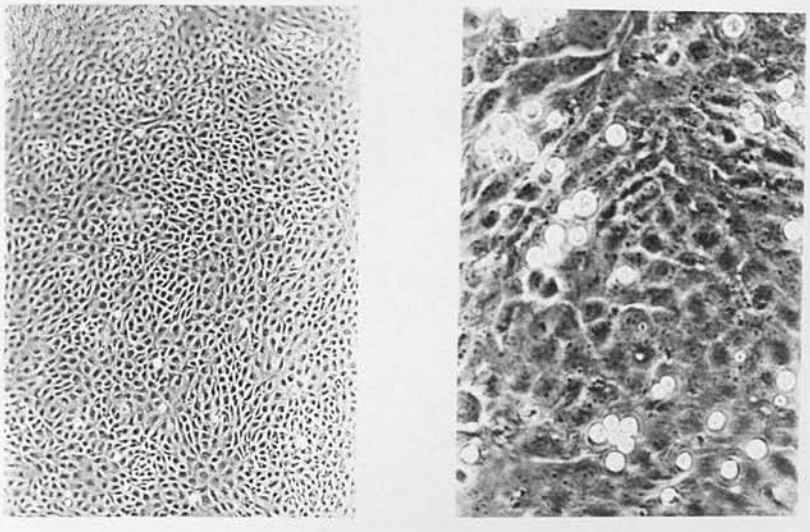


Fig. 3.13b : Rat1A/mycΔ145-262 cells grown to confluence. Cells are polygonal and refractile. Note the abundance of apoptotic bodies.
Phase contrast: Left: x 100; Right: x 320.

3.3.3 Clone 6 cells are Growth Arrested at 32°C.

Exponentially growing cells were shifted to a 32°C incubator. In three independent experiments cells ceased to show increase in cell number at 32°C (Fig. 3.14). There was no increase in apoptosis in the presence of p53 with wild type configuration (see Figs. 3.17 to 3.18 controls). At 37°C, with mutant conformation p53, there was a three-fold increase in cell number over the same period, confirming the original observations of Michalovitz and colleagues (1990). DNA flow cytometry showed both diploid and tetraploid peaks at permissive and nonpermissive temperatures. At 32°C there was in increase in the diploid GO/1 peak (Fig 3.15), and cells did not take up bromodeoxyuridine consistent with this state (data not shown). This growth arrest was reversible by transferring cells to 37°C, even after 2 weeks, or more. By contrast, RcGphe132.4 cells continued to grow at a slightly reduced rate at 32°C in keeping with the previous observations of Michalovitz and colleagues (1990)(data not shown).

3.3.4 Clone 6 cells with Wild-Type p53 are Resistant to both Etoposide and Bleomycin.

At 37°C, in the presence of mutant conformation p53, there was a progressive increase in apoptosis starting 6-10h. after pulsing with drug. The increase was dose dependent: etoposide at 10μM induced a maximum of 6% apoptosis whereas at 50μM the maximum was greater than 30% apoptosis. By contrast, cells maintained at 32°C with p53 in the wild type conformation showed no increase in percentage apoptosis, nor in cell number (Figs. 3.16 and 3.17). Treatment with bleomycin showed similar effects (Fig. 3.18).

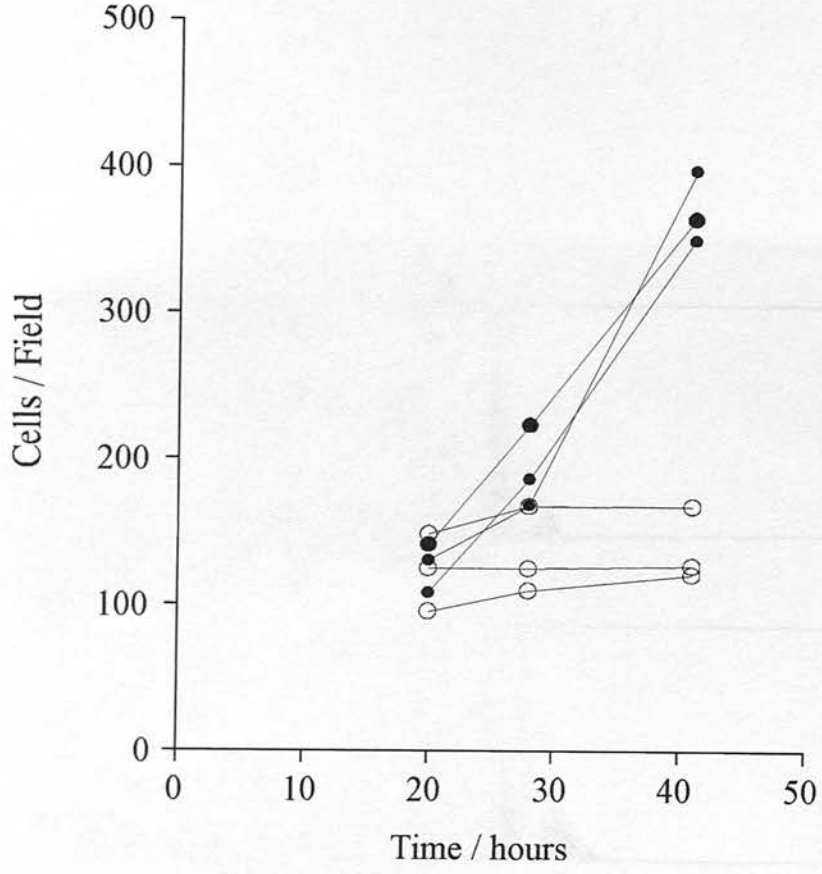


Fig. 3.14 : Growth properties of Clone 6 cells at 37oC (filled circles) and at 32oC (open circles). Each point represents the mean number of cells per field (n=3) in one flask at each time point. Note that the cells incubated at 32oC do not increase in number consistent with a wt p53-induced growth arrest.

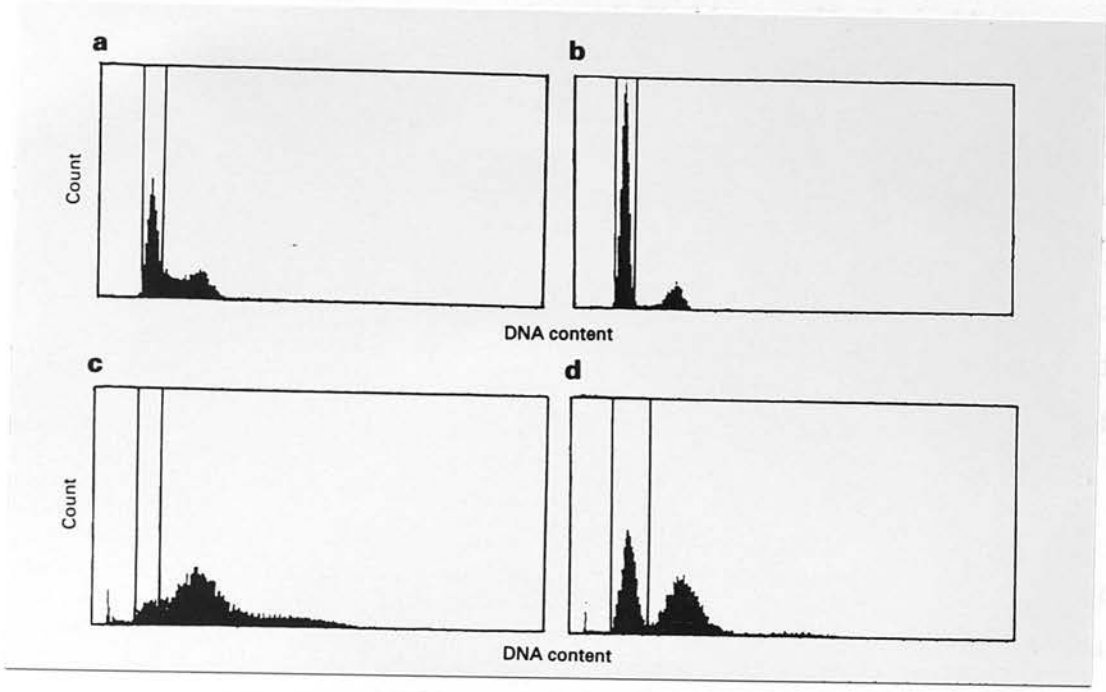


Fig. 3.15. Cell cycle analysis of Clone 6 cells. (**a**) Exponentially growing cells at 37°C, untreated (G0/G1 fraction : 46.56%). (**b**) Following incubation at 32°C for 24 hours, the G0/G1 peak is enlarged (72.51%) and there is a marked decrease in the proportion of cells between the G0/G1 and G2/M peaks (S-phase). (**c**) At 37°C, 24 hours after etoposide treatment (50μM) cells accumulated in G2/M with only 10.18% of cells occupying the G0/G1 position. (**d**) At 32°C, 24 hours after treatment with 50μM etoposide (G0/G1 fraction: 38.51%). Abscissa: DNA content (propidium iodide fluorescence).

RcGphe132.4 cells are sensitive to apoptosis induced by etoposide and bleomycin at 32°C and 37° C: We considered the possibility that these differences in cell proliferation and apoptosis in response to DNA damage might be due simply to altered pharmacokinetics at the different temperatures. The RcGphe132.4 cell line was derived from the same parental stock as Clone 6, but contains a temperature-insensitive mutant p53; hence in this cell line wild type p53 is excluded from function at both 32°C and 37°C. At 37°C Clone 6and RcGphe132.4 cells show closely similar entry into apoptosis: 24h after treatment with 50μM etoposide the incidences were 18.0% and 19.4% respectively. In contrast, at 32°C the incidence of apoptosis in RcGphe132.4 cells was 9.5%, but had fallen to less than 2% in Clone 6 cells. Very similar results were obtained following treatment with bleomycin. At 37°C incidence of apoptosis in Clone 6 cells was 19.8%, but fell to less than 3% at 32°C. In contrast, RcGphe132.4 cells showed 10.3% apoptosis at 37°C and 8.9% at 32°C. Thus the profound inhibition of apoptosis in Clone 6 cells at 32°C is dependent upon the altered configuration of p53 to wild-type and is not explicable solely on the basis of temperature effects on pharmacokinetics.

In order to investigate whether p53-induced growth arrest is capable of preventing apoptosis by allowing time for recovery from DNA damage, cells were incubated at 32°C for 24 hours before treatment with 50μM etoposide, 15μU/ml bleomycin or equivalent volumes of either DMSO or PBS as described above and replacement into the 32°C incubator. At various times after treatment (2,6,12,24 hours), cells were shifted to 37°C. Cells and apoptotic bodies were counted at various time points after treatment. Cells shifted to 37°C 2 hours after treatment with bleomycin showed a similar, though delayed increase in percentage apoptosis. However, after shifting to 37°C after 12 or 24 hours after drug-treatment, no increase in percentage apoptosis was seen (Fig. 3.19). This pattern was also seen with etoposide-treated cells (data not shown). The duration of wild-type p53 expression and the associated G1/S arrest may therefore determine the susceptibility of Clone 6 cells to undergo DNA damage-induced apoptosis.

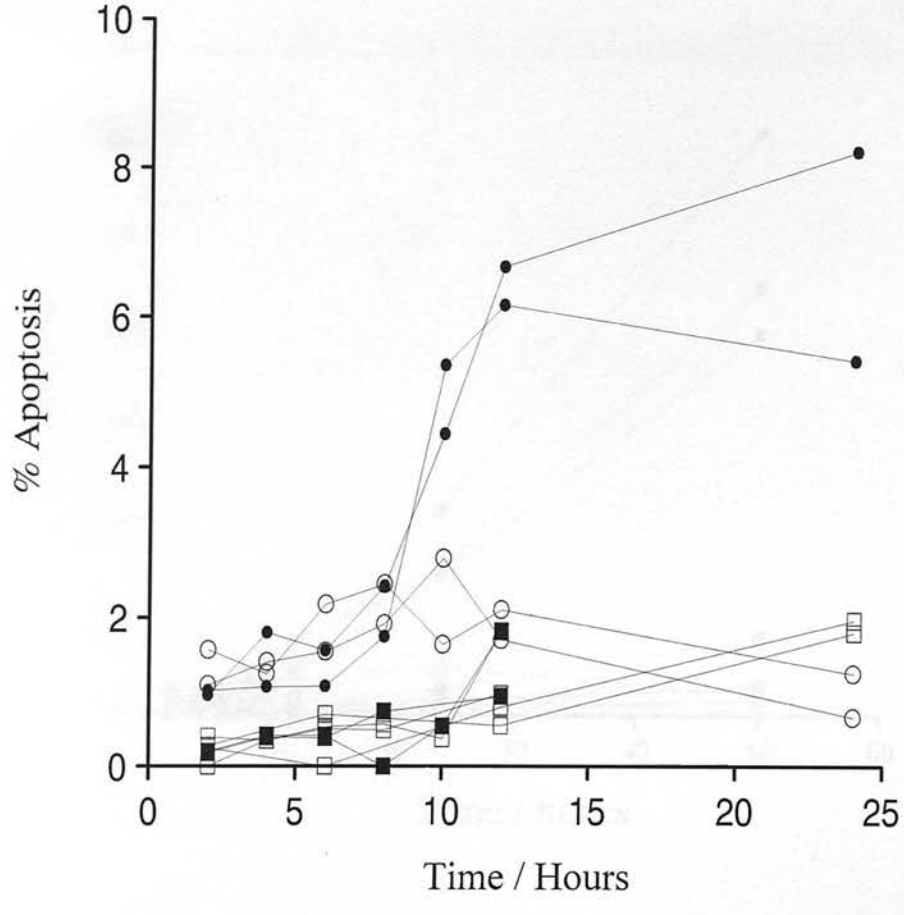


Fig. 3.16 : **Treatment of Clone 6 cells with 10μM etoposide** at 37°C (filled circles) for 1 hour results in substantial apoptosis, whereas treated cells at 32°C (filled squares) and untreated controls (unfilled symbols) do not show this increase. Note the latent period during induction of apoptosis at 37°C. Each line represents a separate experiment (performed in triplicate and expressed as a mean For low values the range was less than 0.6% and for higher values the range was up to 2%).

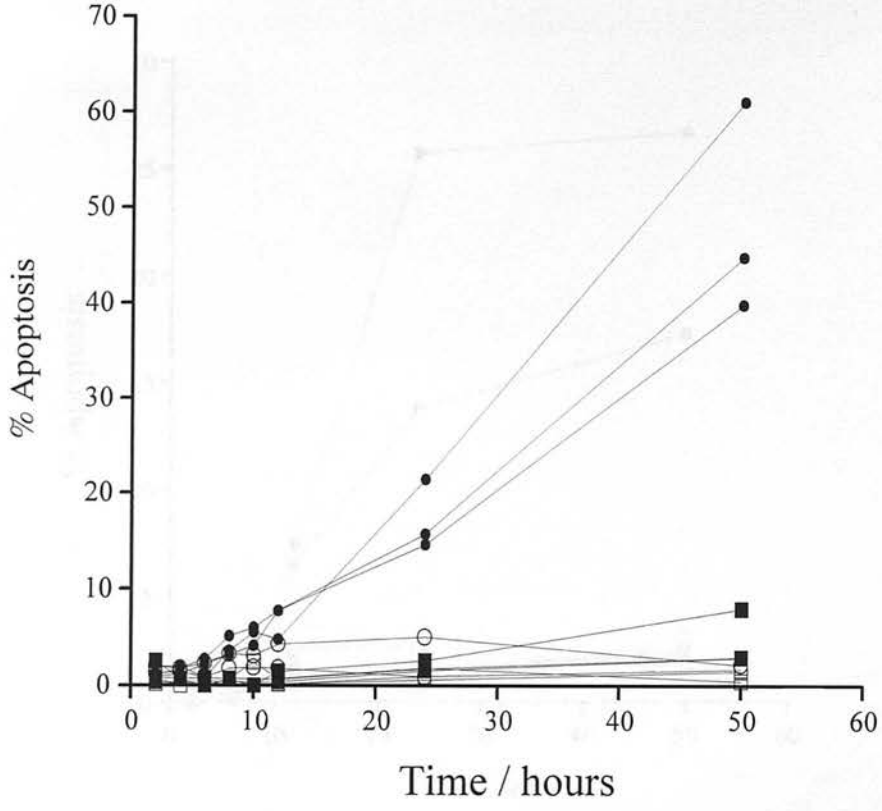


Fig. 3.17 : Treatment of Clone 6 cells with 50μM etoposide for 1 hour induces substantial apoptosis when cells are incubated at 37°C (filled circles). At 32°C treated cells (filled squares) and in control cells treated with an equivalent volume of DMSO vehicle (unfilled symbols) do not show an increase in percentage apoptosis. Each line represents a separate experiment (performed in triplicate and expressed as a mean. For low values range was 2% and for high values was up to 22%).

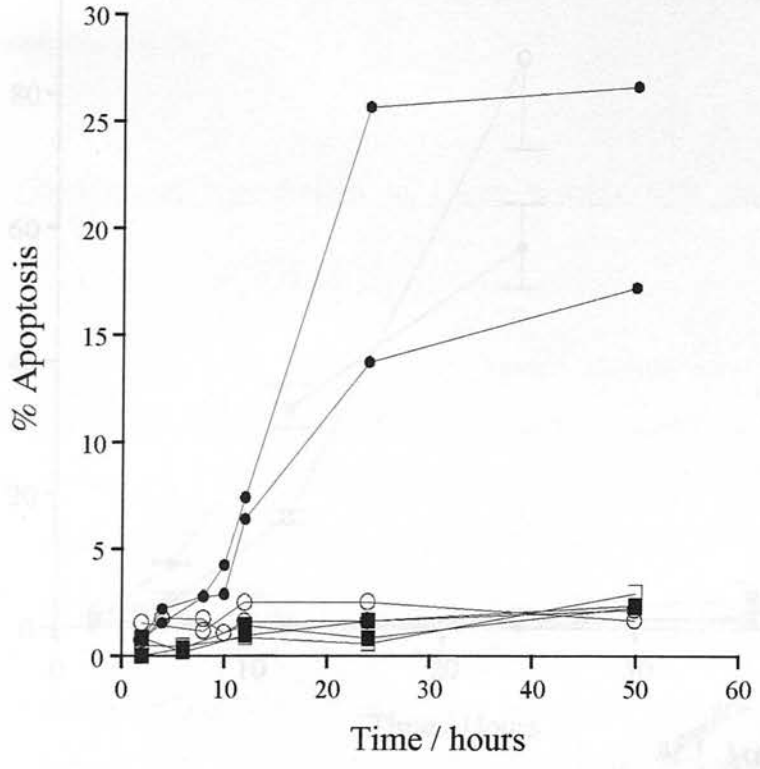


Fig. 3.18 : **Clone 6 cells treated with 15μU/ml bleomycin sulphate** for 1 hour at 37°C (filled circles) and at 32°C (filled squares). Note that treated cells incubated at 37°C undergo substantial apoptosis whereas treated cells at 32°C and untreated controls (open symbols) do not show an increase in percentage apoptosis. Each line represents a separate experiment (performed in triplicate and expressed as a mean. For low values range was 2% and for high values range was up to 12%).

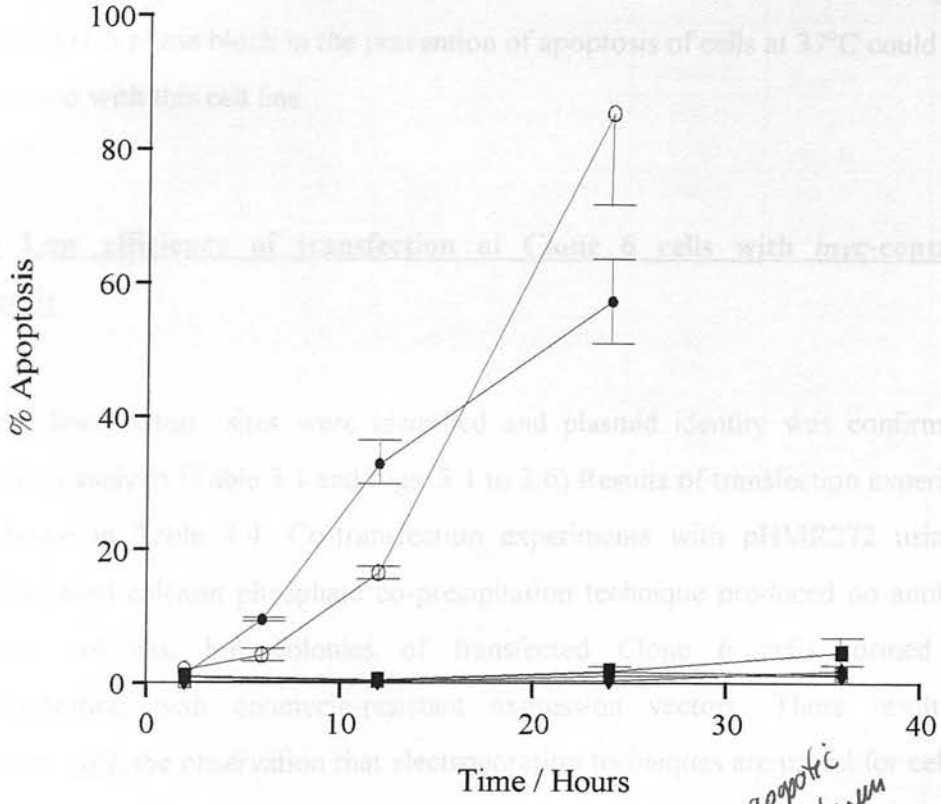


Fig. 3.19 : Clone 6 cells were incubated at 32°C for 24 hours before treating with 15μU/ml bleomycin sulphate for 1 hour. Cells were then incubated for various periods of time at 32°C before shifting to 37°C. Cells incubated at 32°C for 2 hours (filled circles) and 6 hours (unfilled circles) underwent substantial apoptosis after temperature shift. In contrast cells incubated for 12 hours (filled squares), 24 hours (unfilled squares) and untreated controls (filled diamonds) did not show this increase in apoptosis. (mean and SEM; n=3).

L-mimosine (Sigma) was included in the medium at 37°C for 24 hours before and during some drug experiments in order to mimic the reported wild-type p53-induced late G1/S phase block to the cell cycle (Ryan *et al*, 1993; Lalande, 1990). At concentrations reported to induce cell cycle arrest, however, mimosine directly affected cell viability during the time course of these experiments: drug-treated cells underwent apoptosis at rates indistinguishable from control cells. Cells treated at 32°C also showed a loss of viability in the presence of mimosine. As a result, the general role of a G1/S phase block in the prevention of apoptosis of cells at 37°C could not be determined with this cell line.

3.3.5 Low efficiency of transfection of Clone 6 cells with *myc*-containing plasmids

Plasmid linearisaton sites were identified and plasmid identity was confirmed by restriction analysis (Table 3.1 and Figs. 3.1 to 3.6) Results of transfection experiments are shown in Table 3.4. Co-transfection experiments with pHMR272 using the HEPES-based calcium phosphate co-precipitation technique produced no antibiotic-resistant colonies, but colonies of transfected Clone 6 cells formed after electroporation with neomycin-resistant expression vectors. These results are consistent with the observation that electroporation techniques are useful for cell lines which have been found to be refractory to transfection by other techniques (Neumann *et al*, 1982). Only 0.018% of the number of colonies that formed after transfection with the empty vector control plasmid pMV7 were recovered from Clone 6 cells electroporated with pMV7-MER (containing the *myc*ER insert) under identical conditions. These data are consistent with an hypothesis that cells containing both p53val135 and *myc*ER expressed under the control of strong exogenous promoters results in loss of viability of such cells. This suggests that breakthrough gene activity can occur when using temperature-sensitive alleles or oestrogen-sensitive fusion proteins and that this may confound experiments when genes of interest are capable of inducing apoptosis under certain conditions.

but what about those colonies? did they express both transgenes? why did they stop living?

Table 3.4. Transfection Experiments

Expt. No.	Plasmid(s)	Genes‡ Coded	Cell Line	Gene(s)‡ Expressed	Tx Method¶ Selection: μg/ml	Result
1	pLTRp53cGval135 and pHMR272	Ts p53 Hm^r	Rat-1A/mycΔ145-262	active c-myc	$Ca_3(PO_4)$ Hyg-B: 200	No colonies
1	pLTRp53cGval135 and pHMR272	Ts p53 Hm^r	Rat-1A/mycΔ106-143	crippled c-myc	$Ca_3(PO_4)$ Hyg-B: 200	No colonies
2	pLTRp53cGval135 and pHMR272	Ts p53 Hm^r	Rat-1A/mycΔ145-262	active c-myc	$Ca_3(PO_4)$ Hyg-B: 300	No colonies
2	pLTRp53cGphe132 and pHMR272	m p53 Hm^r	Rat-1A/mycΔ145-262	active c-myc	$Ca_3(PO_4)$ Hyg-B: 300	No colonies
2	pLTRp53cGXK and pHMR272	dl p53 Hm^r	Rat-1A/mycΔ145-262	active c-myc	$Ca_3(PO_4)$ Hyg-B: 300	No colonies
2	pLTRp53cGval135 and pHMR272	Ts p53 Hm^r	Rat-1A/mycΔ106-143	crippled c-myc	$Ca_3(PO_4)$ Hyg-B: 300	No colonies
2	pLTRp53cGphe132 and pHMR272	m p53 Hm^r	Rat-1A/mycΔ106-143	crippled c-myc	$Ca_3(PO_4)$ Hyg-B: 300	No colonies
2	pLTRp53cGXK and pHMR272	dl p53 Hm^r	Rat-1A/mycΔ106-143	crippled c-myc	$Ca_3(PO_4)$ Hyg-B: 300	No colonies
3	pMV7-MER	myc-ER Tn5-neo^r	Clone 6	Ts p53 Ha-ras	$Ca_3(PO_4)$ G418: 400	No colonies
3	pMV7	Tn5-neo^r	Clone 6	Ts p53 Ha-ras	$Ca_3(PO_4)$ G418: 400	No colonies
4	pMV7-MER	myc-ER Tn5-neo^r	Clone 6	Ts p53 Ha-ras	EP G418: 400	45 colonies
4	pMV7	Tn5-neo^r	Clone 6	Ts p53 Ha-ras	EP G418: 400	2-3000 colonies

¶Tx = Transfection; $Ca_3(PO)_4$ = calcium phosphate coprecipitation method; EP = electroporation method

‡Ts p53 = (temperature-sensitive) murine p53val135; m p53 = (non-temperature-sensitive) mutant murine p53phe132; dl p53 = p53 deletion (XhoI-KpnI) mutant

Crippled c-myc = mycΔ106-143; Active c-myc: mycΔ145-262

Hm^r: hygromycin-B resistance gene; Tn5-neo^r = neomycin resistance gene from bacterial transposon 5

3.4 Discussion

Expression of wild-type p53 has been shown to induce apoptosis in some cell types (Yonish-Rouach *et al,* 1991; Ryan *et al,* 1993; Shaw *et al,* 1992), G1 arrest and survival in others (Kuerbitz *et al,* 1992; Kastan *et al,* 1991; Mercer *et al,* 1990; Baker *et al,* 1990; Diller *et al,* 1990; Michalovitz *et al,* 1990). In addition, wt p53 has been shown to be an essential intermediate in a signal transduction pathway between the effects of DNA damaging agents (DNA strand breaks) and either apoptosis or G1 arrest (Malcomson *et al,* 1996; Kuerbitz *et al,* 1992; Kastan *et al,* 1992; Lowe *et al,* 1993; Clarke *et al,* 1993). In this way p53 seems to play a critical role in deleting certain cell types that have sustained DNA damage (eg. thymocytes (Clarke *et al,* 1993), lymphocytes (Gottleib *et al*, 1994; Howie *et al*, 1994b) and myeloid progenitor cells (Lotem and Sachs, 1993)) or in establishing a state of G1 arrest, possibly permitting DNA repair (Bakalkin *et al,* 1994; Lane, 1993).

The finding that p53 function is lost in many authentic human and experimentally induced animal tumours has led to the assumption that p53 loss of function is causally associated with resistance to anticancer therapy (Lowe *et al,* 1993a). In this study, the importance of p53 status on the sensitivity of cells to apoptosis induced by two anticancer drugs and by co-expression of c-*myc* was addressed.

In this work, a fibroblast cell line transformed with activated Ha-*ras* and temperature-sensitive p53 transgenes was used to show that wild type p53 can lead to G1 arrest and at the same time resistance to the DNA damaging agents bleomycin and etoposide. By contrast, in the presence of mutant conformation p53, cells underwent apoptosis associated with a relative accumulation in G2/M, a common response to DNA injury in yeast and mammalian cells (Hartwell and Weinert, 1989). Mimicry of a G0/G1 arrest in Clone 6 cells at 37°C by either mimosine treatment or serum starvation was not informative in the case of Clone 6 cells as these treatments caused the death of the cultures. It was therefore impossible, using this cell line, to

show directly that a growth arrest in G0/G1, independent of p53, was protective against DNA damage.

These findings apparently contrast with published work in which temperature-sensitive p53 was expressed in the M1 myeloid leukaemic (M1; Yonish-Rouach *et al,* 1993) and murine erythroleukaemic (MEL; Ryan *et al,* 1993) cell lines where apoptosis was induced upon incubation at 32°C (ie. with wild-type p53). MEL cells underwent G1 arrest prior to undergoing apoptosis, but in M1 cells, no growth arrest could be observed at any position in the cell cycle. Other cell types (including rat fibroblasts) have been shown to undergo G1 arrest but not apoptosis in response to wild-type p53 induction (Michalovitz *et al,* 1990; Diller *et al,* 1990; Mercer *et al,* 1990; Kastan *et al,* 1992). In cell lines derived from clinically-sensitive human tumours, DNA-injury induced wild-type p53 was held to be responsible for decreased clonogenicity following ionising radiation and this effect could be reversed by transfection of a dominant negative mutant p53. (McIlwrath *et al,* 1994). While bleomycin and etoposide maximally kill cells in S phase, where replication forks are forced to negotiate either cleaved complex / double strand breaks (etoposide; Bae *et al,* 1988) or double-strand breaks resulting from free radical attack (bleomycin; Kuo, 1981), they can damage and kill cells in G0/G1 (Clarke *et al,* 1993; Roy *et al,* 1992; Evans *et al,* 1994). The simplest explanation of the data presented here is that the G1 arrest mediated by p53 facilitates survival of *ras*-transformed fibroblasts by allowing effective DNA repair and prevents entry into S phase, a stage when cells are often most susceptible to DNA damage.

Depending upon the cell system chosen, induction of p53 can cause either G1 arrest, apoptosis or both apoptosis and G1 arrest (Michalovitz *et al,* 1990; Ryan *et al,* 1993; Yonish-Rouach *et al,* 1993; Debbas and White, 1993; Wu and Levine, 1994). The mechanisms by which decisions are taken that favour either of these endpoints are poorly defined but these decisions can be affected by specific growth factors (Yonisch-Rouach *et al,* 1991; Gottleib *et al,* 1994; Canman *et al,* 1995). In particular, it is not known how p53 can mediate apoptosis in the thymocyte but not in the

fibroblast. The recognition of DNA damage (possibly involving the ataxia telangiectasia gene products; Kastan *et al,* 1992) leads, via p53, to the control of the cell cycle at the G1 checkpoint. We have shown this pathway to be protective in fibroblasts. Our results complement that of Lowe *et al,* (1993) who showed that p53-normal fibroblasts were susceptible to anticancer treatment as a result of abrogation of the p53-mediated G1 arrest by adenovirus EIA expression. Further, IL6 protects M1 cells from undergoing p53-mediated cell death (Yonish-Rouach *et al,* 1991, 1993) and this protection also correlates with the induction of a stable G0/G1 arrest. This supports the supposition that activation of wild-type p53 can result in different response depending upon other modifying factors such as cell lineage and the trophic environment. (Levy *et al,* 1993).

Using a different mutated p53 (proline substituted at residue 193) under its physiological promoter, Bristow and colleagues (1994) have shown that co-transfection of activated Ha-*ras* and mutated p53 into a primary rat embryonal fibroblast cell line resulted in enhanced clonogenicity *in vitro* and tumorigenicity in SCID mice after irradiation compared with cell lines containing *ras* alone. This effect was dependent on the level of mutant p53 expression, presumably as a result of competition with endogenous wild type p53. However they did not directly assess the proportion of cells undergoing proliferation, growth arrest or cell death. Experiments similar to those in Bristow *et al,* (1994) with ionising radiation sources could not be carried out here as it was found that reproduciblity of results could not be maintained if there were fluctuations in temperature of Clone 6 cells before or during experiments. Indeed, clonogenicity of Clone 6 at 32°C was negligible.

Our *in vitro* experiments with DNA damaging drugs (including the radiomimetic bleomycin) show that, under certain circumstances, overexpression of wild type p53 can protect a cell which has suffered DNA injury against death rather than kill it, by causing the cell to growth arrest in G1. The corollary *in vivo* is that wild type p53 in an appropriate cellular context could confer a state of increased drug resistance. The significance of mutated p53 oncosuppressor gene in clinical drug resistance is likely

to be both complex and variable depending on the existence of other pathways of cell cycle activity control and response to injury.

Very recently, a study of tetracycline-inducible p53 in p53 null immortalised mouse embryonic fibroblasts revealed an slight anti-apoptotic activity of p53 that was only apparent at very low levels of p53 expression (Lassus *et al,* 1996). However the authors drew a distinction between a protective effect of the enforced growth arrest caused by overexpression of wild-type p53 seen here (published as Malcomson *et al,* (1995)) and their protective effect that was elicited without sufficient p53 expression to cause growth arrest. The basis of this effect is not known.

It was shown here that death of fibroblasts induced by etoposide and bleomycin occurs independently of wild-type p53 function. This confirms work by Strasser *et al,* (1994) which showed that thymic lymphoma cells from p53 -/- mice underwent apoptosis by p53-independent mechanisms following irradiation. Tumours which contain cells with mutated p53 initially may be more susceptible to cell death caused by therapy. However in the absence of G1 arrest caused by wild type p53, and therefore in the presence of continuing cell cycle activity, combined with karyotype instability (Yin *et al,* 1992; Livingstone *et al,* 1992), clones resistant to therapy may appear thus conferring a clinical state of "drug resistant" disease.

Waf1 (Cip1 / Sid1, p21), a gene product which is induced by wt p53, has potent inhibitory activity on cyclin E / cdk2 complexes in cells undergoing radiation-induced G1 arrest (Dulic *et al,* 1994; ElDeiry *et al,* 1994; ElDeiry *et al,* 1993). Waf1 is therefore a major regulator of cell cycle progression at the G1/S interface. The expression of Waf1 in cell types that undergo apoptosis following activation of the p53 pathway has suggested that it may be active in both arrest and death mechanisms. The decision of a cell to die may therefore be determined by other lineage-dependent messages or growth factors (Canman *et al,* 1995). Recently, it has been shown that apoptosis of thymocytes from $p21^{WAF1\ -/-}$ mice underwent normal p53-dependent apoptosis following irradiation. Therefore it is unlikely that p53 mediates apoptosis

through its transcriptional upregulation of p21. Further, these mice do not show an increased rate of neoplasia similar to that suffered by the p53 -/- mice (Deng *et al,* 1995; Brugarolas *et al,* 1995).

It therefore seems that the growth-arresting property of p21 is not the chief mechanism of p53-medated tumour suppression. Other functions of p53, including apoptosis, may therefore be critical for tumour suppression by p53. Moreover, emerging data from the analysis of various activities of p53 mutants has demonstrated that induction of apoptosis and suppression of transformation (in suitable test cell lines for each function) by p53 can be separated from transcriptional activation of p21 and growth arrest. It appears, however, that some of these mutants that are defective for induction of apoptosis (but not for induction of p21 expression) are nevertheless unable to activate transcription from the Bax or IGF-binding protein-3 (IGF-BP3) promoters (Rowan *et al,* 1996; Friedlander *et al,* 1996; Ludwig *et al,* 1996). It has been postulated that these and other downstream transcriptional targets of p53 (such as Fas (OwenSchaub *et al,* 1995)) mediate its apoptotic function. However, this is not necessarily the case in all cellular situations as evidenced by the normal p53-dependent apoptotic response in Bax -/- mice (Knudson *et al,* 1995) and by observations that p53 mediated apoptosis can occur without detectable changes in Bax expression (Allday *et al,* 1995; Canman *et al,* 1995; Rowan *et al,* 1996). However, pathways of gene activation leading from p53 to apoptosis may be redundant and this may explain differential usage of such pathways in various cellular contexts. Nevertheless, a p53 protein that has no transcriptional activity as a result of the so-called 22/23 mutation in the *trans*-activation domain has been used to show that, in rodent kidney cells, transcriptional activation by p53 is essential for induction of apoptosis, whereas in HeLa cells *trans*-activation by p53 is entirely dispensable (Haupt *et al,* 1995; Sabbatini *et al,* 1995).

Clearly, the cellular context in which p53 is expressed is important for determining the consequences of p53 activation within a given cell type. As discussed above, the growth factor milieu and cell lineage have important bearings upon this. In addition

to factors that affect general susceptibility to apoptosis, such as the Bcl-2-related proteins (Guillouf *et al,* 1995; Chiou *et al,* 1994; Wang *et al,* 1995; Schott *et al,* 1995; Debbas and White, 1993) expression of mitogenic oncogenes appears to alter the susceptibility to p53-mediated apoptosis. The Clone 6 cells used for this study were transformed with activated c-Ha-*ras.* It is known that ras-overexpression can inhibit responses to apoptotic stimuli (Lin *et al,* 1995; Arends *et al,* 1993)and may therefore play a part in determining the response to wild-type p53 in this cell line. However, the presence of *ras* does not appear to affect p53-independent apoptosis of Clone 6 cells at 37°C in response to bleomycin or etoposide. Of particular note, on the other hand, is the observation made here that serial transfection of *myc*-ER and p53val135 into rat fibroblasts did not result in stable expression, either in the presence or absence of activated *ras* expression. After initial slow colony formation, such transfectants were not expandable into stable cell lines. This is a common observation in experiments where cells are transfected with plasmids containing constitutively-expressed genes that cause apoptosis (Lowe *et al,* 1993a). Given that complete control of the temperature-sensitive p53 and *myc*-ER activities is unlikely (M.Oren, personal communication; Danielian *et al,* 1993; Berry *et al,* 1990) , even under the cell culture conditions used, this observation is consistent with the hypothesis that p53 and c-*myc* co-operate to induce apoptosis in cells that do not normally undergo apoptosis in response to wild-type p53 induction. This is supported by observations that apoptosis induced by serum starvation of c-*myc*-transfected mouse embryo fibroblasts is dependent upon the presence of functional p53 alleles (Hermeking and Eick, 1994; Wagner *et al,* 1994). Likewise, overexpression of the transcriptional regulator E2F-1 that is involved in cell cycle progression into S phase can co-operate with wild type p53 to induce apoptosis (Wu and Levine, 1994) and the apoptosis inducing functions of both E2F-1 and the adenovirus E1A oncogene are blocked by disruption of both p53 alleles (Lowe *et al,* 1994; Kowalik *et al,* 1995).

The inability to produce stable cell lines expressing both c-*myc* and wild-type p53 by the methods used in this chapter made it impossible to confirm, at that stage, speculation that co-expression of these two genes was synergistic and could trigger

apoptosis by default under normal culture conditions. It was thought possible that these assumptions could be tested *in vitro* if greater control was exerted over the expression of apoptosis-promoting genes at the transcriptional, rather than at the post-translational level. Therefore, inducible expression systems were evaluated for use with apoptosis-promoting genes as described in the following chapter.

Chapter 4.

4. Evaluation of Inducible Expression Systems for the Study of Apoptosis-Related Genes.

4.1 Introduction

As discussed in the previous chapters, generation of stably-transfected cell lines constitutively expressing genes that force cells to undergo either a cell cycle arrest or apoptosis is not possible. In the situation described in Chapter 3, where several attempts were made to produce cell lines co-expressing a temperature-sensitive p53 mutant and *myc*-ER, it was surmised that the leak-through activity, however small, from both these transgenes was sufficient to have a profound effect upon cell viability. The inability to produce such lines made it difficult to investigate a possible co-operative interaction between p53 and c-*myc* using this approach. Such an interaction would be significant, given the high prevalence of alterations of both these genes in many human cancers. Detailed analysis of such an interaction could provide valuable information regarding the relationship between cell cycle control and apoptosis. It was therefore decided to persist with attempts to generate such lines, but by using a different strategy. This was to utilise the properties of inducible gene expression systems so that the transcription of the test gene mRNAs could be kept as low as possible during transfection, selection and cloning of stable cell lines. Induction of the test genes could then be controlled by the addition of an exogenous chemical, ideally without toxic side-effects affecting the expression of endogenous genes. A survey of available inducible expression systems was carried out, the results of which appear in Chapter 1. Two systems were investigated. The first, the Lacswitch system based upon the lac operator-repressor interaction was chosen

because of the high affinity of the lac repressor for its cognate recognition sequence; the low toxicity and rapid uptake of its inducer, IPTG; and for the ability of lacI to greatly decrease expression from a strong viral promoter whilst retaining inducibility of up to 100 fold. The second system, involving the VP16GalER^{tm} tamoxifen-dependent transcriptional activator, was chosen so that a comparison could be made between repression of an otherwise constitutive promoter in the absence of inducer and specific activation of a synthetic promoter occurring only in the presence of exogenous ligand. In order to make the testing of these two systems as stringent as possible for the purpose of investigating apoptosis genes, both of these systems were used to control expression of mouse Nedd2, an ICE-like protease known to cause apoptosis, in addition to the development of similar vectors for the expression of c-*myc*, p53 and p21$^{WAF1/CIP1}$.

4.2 Materials and Methods

4.2.1 Vector Construction

Detailed vector maps for intermediates in construction strategies are given in alphabetic order in Appendix C.

4.2.1.1 .Alterations to Existing Expression Vectors.

4.2.1.1.1 Lac Vectors

The CAT gene was removed from pOPRSVICAT by digestion with NotI and recircularisation with T4 DNA polymerase to generate pOPRMT. The G418 resistance cassette in pOPRMT was replaced in pOPRMTBglII by a BglII linker following digestion with AatII and SacI and treatment with T4 DNA polymerase. The puromycin resistance cassette from pPUR (de la Luna, 1988) was cloned into the BglII site of pOPRMTBglII as a 1.4kb PvuII/BamHI fragment (pOPRpuro). Because of a difficulty in preparing sufficient quantities of plasmid pPUR for transfection experiments, plasmid pPURO (Fig. 4.14) was derived from pOPRpuro by removal of the RSV LTR by digestion with BstXI and NotI and recircularisation following treatment with T4 DNA polymerase. The NotI site was regenerated. pPURO plasmid contains the ampicillin resistance gene and ColE1 origin of replication, both derived from pBluescript and appears to replicate more efficiently in XL1BlueMRF'cells than pPUR, which is based upon pBR322, a lower copy number plasmid.

The NotI site of pOPI3CAT was converted to a BclI site in pOPI3N-BclI. This was achieved by linearisation of pOPI3CAT with NotI, blunt-ending with T4 DNA polymerase and adding phosphorylated BclI linkers. As BclI restriction is inhibited by *dam* methylation, pOPI3N-BclI DNA was maintained in the *dam*-deficient *E. coli* strain SCS110. Plasmid pOP13N-BclI contains an intron containing three *lac* operator sites in a 485bp BglII/BclI fragment.

4.2.1.1.2 Gal-4 Responsive Vectors

Versions of pSP65GC (Figs. 2.6 and 4.1a) were made that provided either puromycin (pSP65GCpuro) or hygromycin (pSP65GChygro) resistance. The puromycin resistance (PAC) gene under the control of an SV40 early region promoter/enhancer and followed by an SV40 polyadenylation signal was excised from pPUR (also referred to as pBSpacΔP) (de la Luna, 1988) using PvuII and BamHI, blunt-ended with T4 DNA polymerase and subcloned into a blunt-ended SphI site of pSP65GC. The orientation of the puromycin resistance cassette in pSP65GCpuro was determined relative to the Gal4-responsive promoter by digestion with PstI which produced a 1068bp fragment. Similarly, a hygromycin resistance gene under the control of phosphoglycerate kinase (PGK) gene control elements contained within a 1960bp BglII fragment was subcloned blunt-ended from pPGKhygro into the blunted SphI site of pSP65GC. Orientation of the hygromycin resistance cassette in pSP65GChygro was confirmed by digestion with EcoRI (586bp) (Figs 4.1b,c and 4.2).

4.2.1.2 p53 Plasmids

In order to generate a wild-type murine p53 cDNA that could be easily subcloned into expression vectors, p53 sequences from two plasmids were combined. The first plasmid, pSV53C (Jenkins et al, NAR 12 1994), which contains a full length, murine pseudo-wild-type p53 cDNA from bases 19 to 1377 (numbered according to GenEMBL x00741) in an SV40 expression cassette. This sequence was originally cloned as pP53-5 from an SV40-transformed murine fibroblast cell line (SVA31E7) and contains 4 mutations in the non-conserved 5' end of the coding sequence: Arg48 (CGA), Gln79(CAG), Trp80(TGG), del 81. The second plasmid, pMSVcl-ala (Finlay et al, 1988) contains a wild-type murine p53 cDNA.

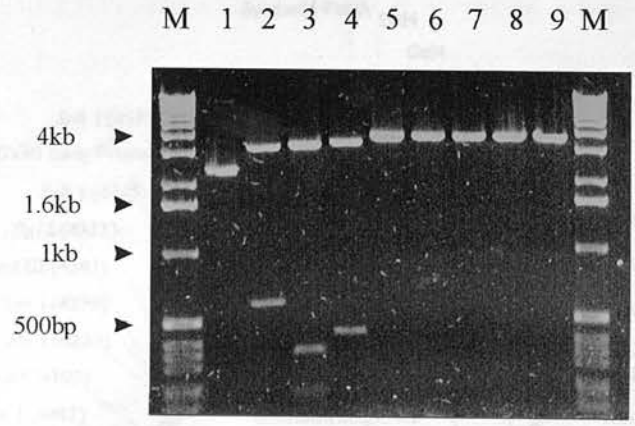


Fig. 4.1a: pSP65GC. M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: SalI; Lane 6: EcoRV; Lane 7: PstI; Lane 8: PvuII; Lane 9: SphI.

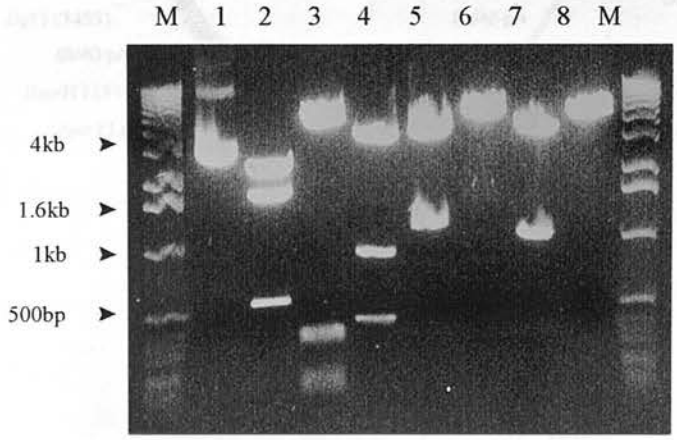


Fig. 4.1b: pSP65GCpuro. M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: SalI; Lane 6: EcoRV; Lane 7: PstI; Lane 8: PvuII.

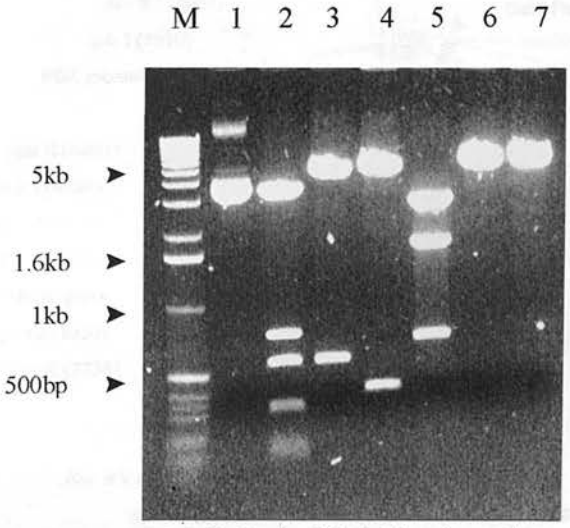


Fig. 4.1c: pSP65GChygro. M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: EcoRV; Lane 6: SalI; Lane 7: PvuII.

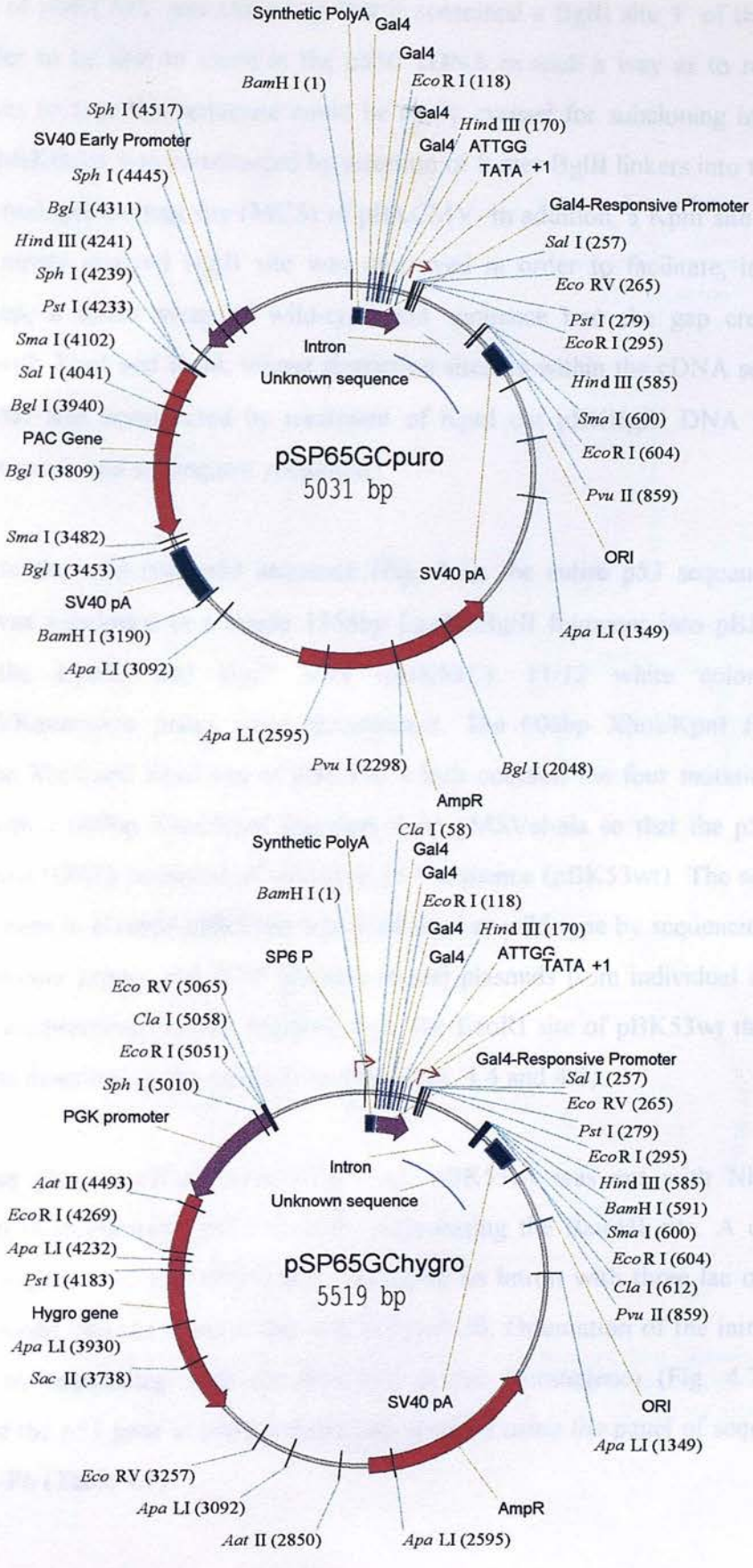


Fig.4.2: pSP65GCpuro and pSP65GChygro. AmpR: prokaryotic ampicillin resistance gene. pA, PolyA: polyadenylation signal. PAC: puromycin resistance gene. PGK: phosphoglycerate kinase.

The MCS of pBKCMV was altered so that it contained a BglII site 3' of the EcoRI site in order to be able to clone in the p53C cDNA in such a way as to retain the cloning sites so that this sequence could be easily excised for subcloning into other plasmids. pBKBglII was constructed by insertion of 8-mer BglII linkers into the SmaI site in the multiple cloning site (MCS) of pBKCMV. In addition, a KpnI site that lies 3' of the newly inserted BglII site was destroyed in order to facilitate, in a later cloning step, a direct swap of wild-type p53 sequence into the gap created by digestion with XhoI and KpnI, whose restriction sites lie within the cDNA sequence. pBKBglIIΔK was constructed by treatment of KpnI cut pBKBglII DNA with T4 DNA polymerase and subsequent religation.

To generate the wild type p53 sequence (Fig. 4.3), the entire p53 sequence from pSV53C was subcloned in a single 1358bp EcoRI/BglII fragment into pBKBglΔK between the EcoRI and BglII sites (pBK53C). 11/12 white colonies on Xgal/IPTG/Kanamycin plates were recombinant. The 606bp XhoI/KpnI fragment between the XhoI and KpnI site of pBK53C which contains the four mutations was replaced with a 609bp XhoI/KpnI fragment from pMSVcl-ala so that the p53 open reading frame (ORF) consisted of wild-type p53 sequence (pBK53wt). The sequence of the p53 gene in plasmid pBK53wt was confirmed as wild-type by sequencing (with the T3 promoter primer and dITP labelling mixes) plasmids from individual colonies containing unidirectional nested deletions from the EcoRI site of pBK53wt that were generated as described in the methods section (Figs. 4.4 and 4.5).

To generate plasmid pBKOP53wt (Fig. 4.6), pBK53wt was cut with NheI and BamHI and recircularised (pBK53wtN/B) regenerating the BamHI site. A c.400bp BglII/BclI fragment from pOPI3N-BclI containing an intron with three lac operator sites was cloned into the BamHI site of pBK53wtN/B. Orientation of the intron was confirmed by sequencing with the BKCMV primer (Stratagene) (Fig. 4.7). The sequence of the p53 gene in pBKOP53wt was checked using the panel of sequencing primers P1-P6 (Table 4.1).

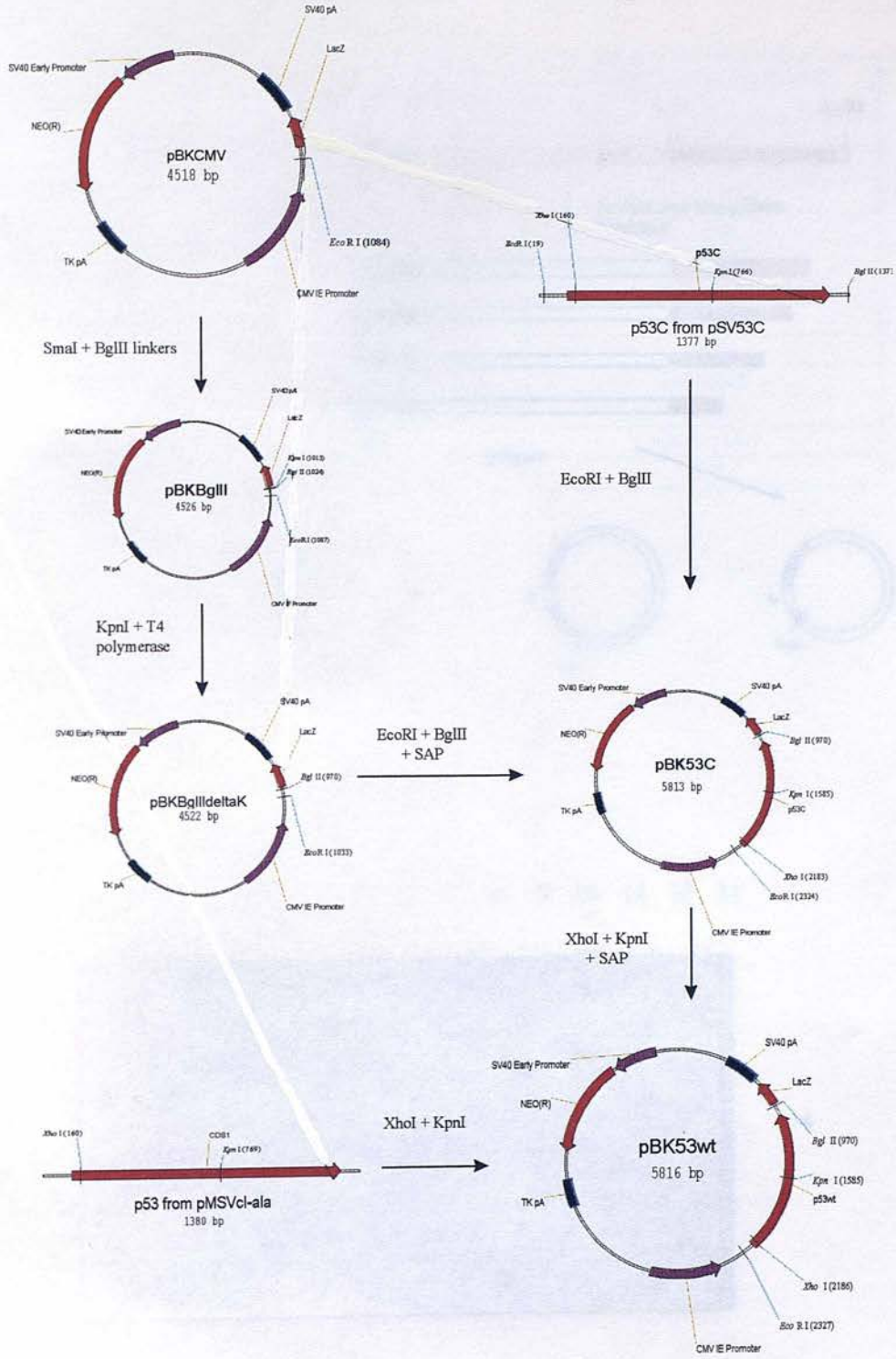
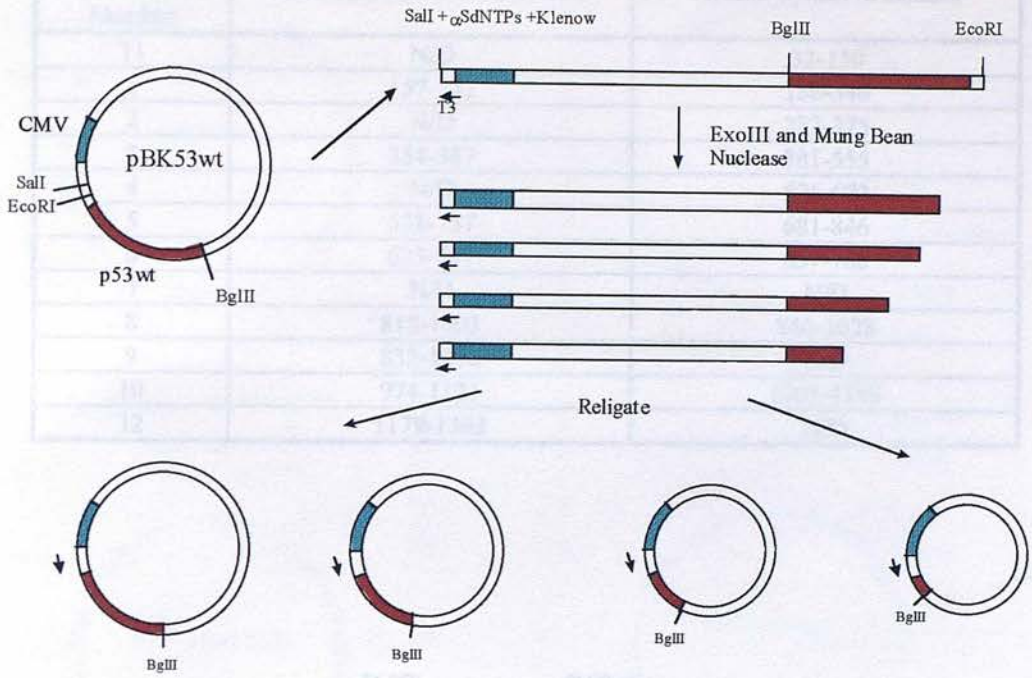


Fig. 4.3: Construction strategy for pBK53wt. (Detailed maps for intermediates in Appendix C.) NEO (R): G418 and kanamycin resistance gene. pA: polyadenylation signal. CMV IE: cytomegalovirus intermediate early region. LacZ: *lacZ* α-peptide gene. SAP: Shrimp alkaline phosphatase. TK: *Herpes simplex* virus thymidine kinase.

A



B

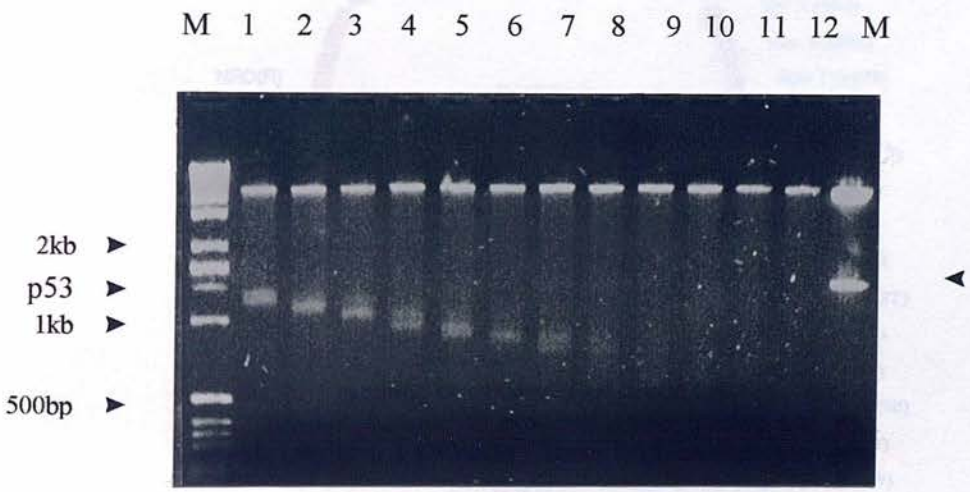


Fig. 4.4. A). Strategy for the generation of unidirectional nested deletions from the EcoRI site of pBK53wt. CMV: CMV promoter. Arrows: T3 promoter primer binding site. **B).** Generation of deletions of pBK53wt. M: Life Tech 1 kb ladder. Lanes 1-12: increasing lengths of time (in 10 second intervals) of digestion with exonuclease III. Arrow: kb ladder spiked with undigested EcoRI/BglII cut pBK53wt

Deletion Number	Bromophenol Blue to Bottom	Xylene Cyanol to Bottom
11	N/D	32-150
1	157-292	188-346
2	N/D	237-375
3	354-387	381-555
4	N/D	526-672
5	638-757	681-846
6	623-797	657-766
7	N/D	N/D
8	818-1003	846-1028
9	832-1070	N/D
10	974-1121	1005-1196
12	1179-1362	N/D

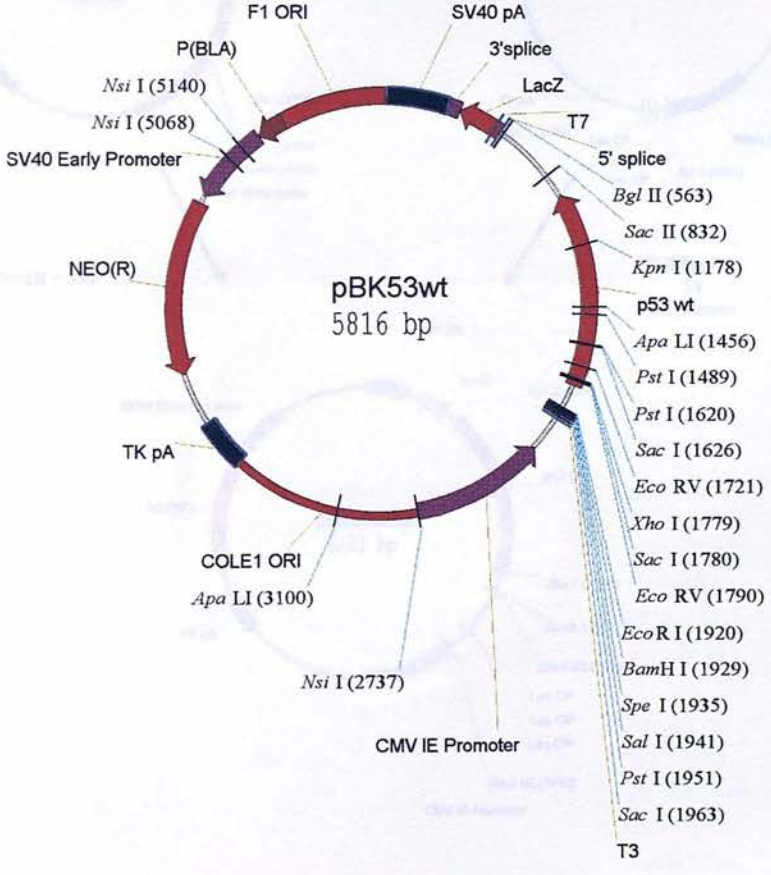


Fig. 4.5: pBK53wt (Bottom). Labels as in previous figures. **Table**: Regions of the p53 gene in pBK53wt sequenced using clones containing nested deletions.

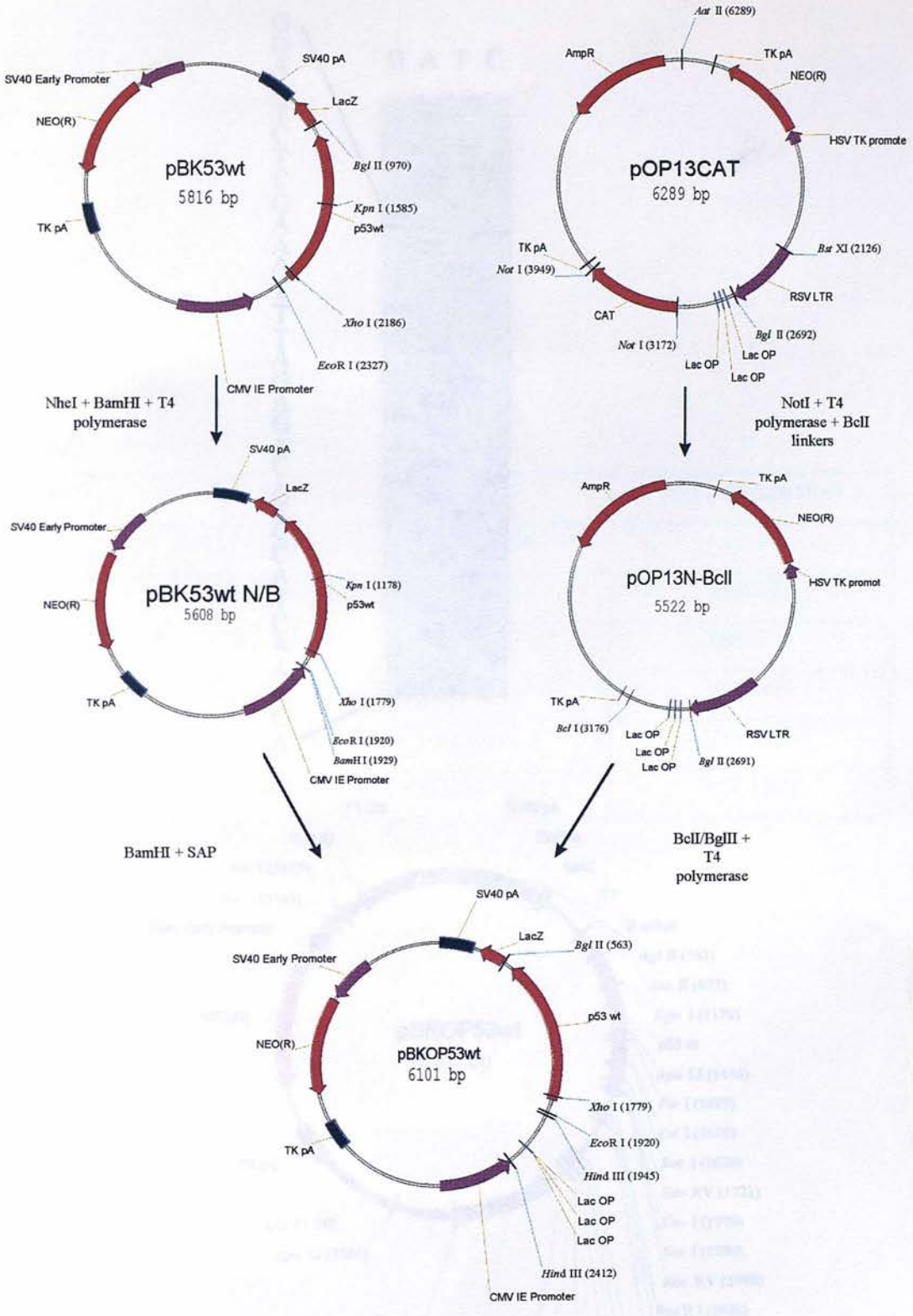
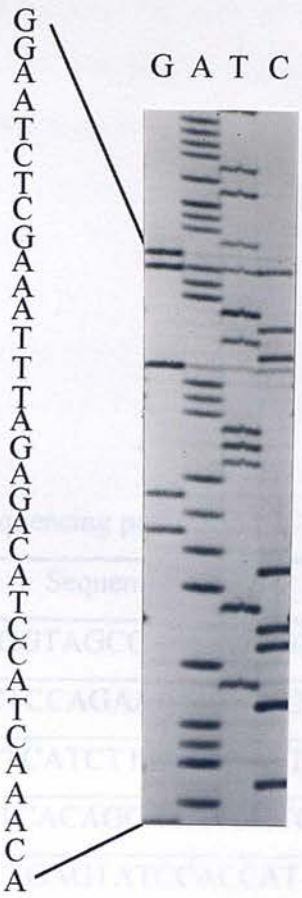


Fig. 4.6: Construction strategy for pBKOP53wt. (Detailed maps for intermediates in Appendix C.) Lac OP: ideal *lac* operator site. Other labels as for previous figures.



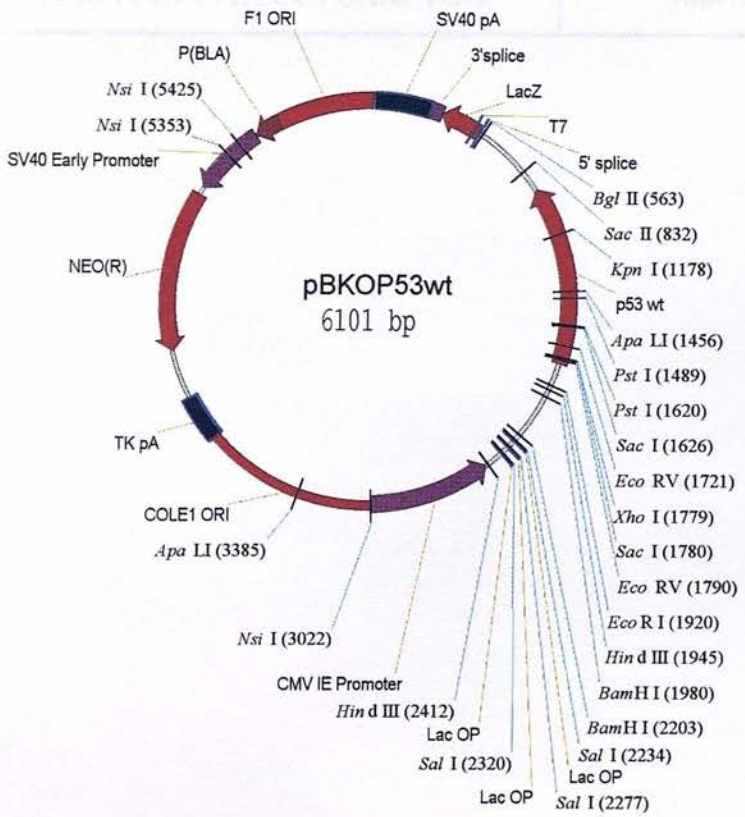


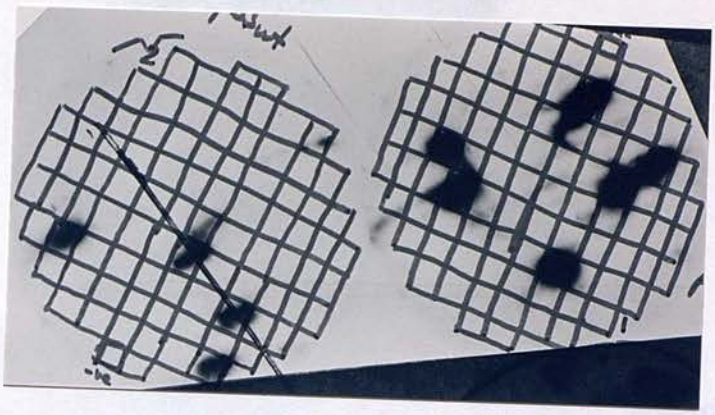
Fig. 4.7. pBKOP53wt. Top: Sequencing of pBKOP53wt mini-prep sample 4 with BKCMV primer showing part of the *lac* operator-containing insert sequence. The orientation of the insert relative to the eukaryotic promoter is the same as in pOPI3CAT.

Table 4.1. Mouse p53 sequencing primers.

Primer	Sequence	p53 position (bp)
P1	5'-CTGTAGGTAGCGACTCACGT-3'	32-41
P2	5'-CTTCCTCCAGAAGATATCC-3'	207-225
P3	5'-CCCTGTCATCTTTTGTCCCT-3'	385-404
P4	5'-AGAAGTCACAGCACATGACG-3'	601-620
P5	5'-GGCTCTGAGTATCCACCAT-3'	786-805
P6	5'-AAGTCCTTTGCCCTGAACTG-3'	988-1007

To generate plasmid pOPRp53wt (Fig. 4.8), pBK53wt was linearised with EcoRI and BglII, blunt-ended with T4 DNA polymerase and ligated to 10-mer phosphorylated NotI linkers. Following digestion with NotI the 1376bp fragment was isolated from a 1% agarose gel and ligated to NotI cut, dephosphorylated pOPRMT. Orientation of the p53 insert was checked by digestion with SacI which generated a 1167bp fragment. Plasmid pSP65GCp53wt was constructed by blunt-end subcloning of the 1.4kb EcoRI/BglII fragment from pBK53wt into the EcoRV site (treated with T4 DNA polymerase) of pSP65GC. To generate a plasmid (pSP65GCp53wtpuro) which contains a puromycin resistance cassette in the opposite orientation to the Gal4-p53wt expression cassette a single blunt-ended 1.4kb PvuII/BamHI fragment from pPUR (de la Luna, 1988) into the SphI site (treated with T4 DNA polymerase) of pSP65GCpuro. All fusion sites were destroyed in the cloning procedure. The orientation of the puromycin resistance cassette was determined by digestion with PstI which produced a 915bp diagnostic fragment. To generate a Gal4-responsive p53 expression vector containing a hygromycin resistance cassette, a blunt-ended BglII fragment of approximately 1960bp in length was subcloned from pPGKhygro into the SphI site (blunt-ended with T4 DNA polymerase) of pSP65GCp53wt. Orientation of the hygromycin cassette was determined by digestion with EcoRI (Fig. 4.9 and 4.10).

A



B

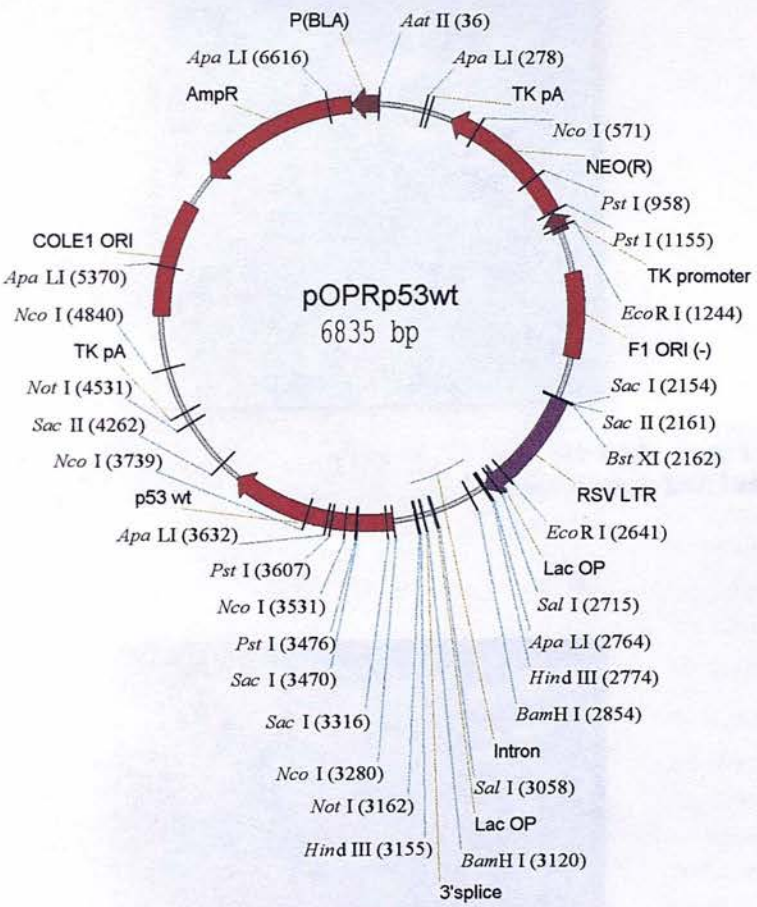


Fig. 4.8. A) Colony hybridisation screen for pOPRp53wt; p53 cDNA probe. **B)** pOPRp53wt.

Fig. 4.9a. pSP65GC53wthygro mini-preps: Lanes 1-6 (2 correct), EcoRI. **pSP65GChygro mini-preps**: Lanes 7-12 (8 and 12 correct), EcoRI.

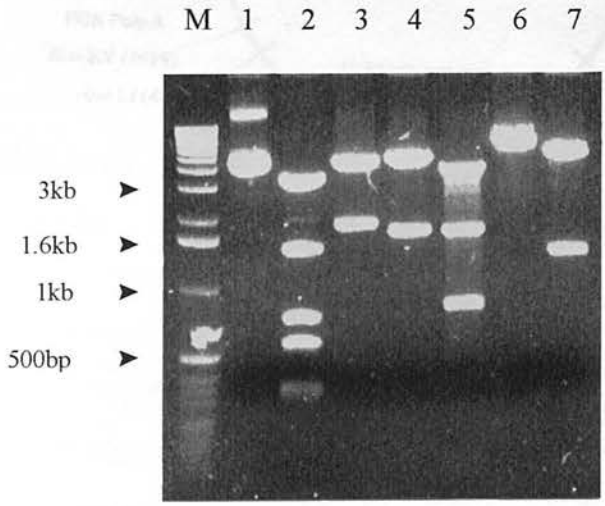


Fig. 4.9b. pSP65GC53wthygro maxi-prep DNA.M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: EcoRI; Lane 3: BamHI; Lane 4: HindIII; Lane 5: EcoRV; Lane 6: SalI; Lane 7: PvuI.

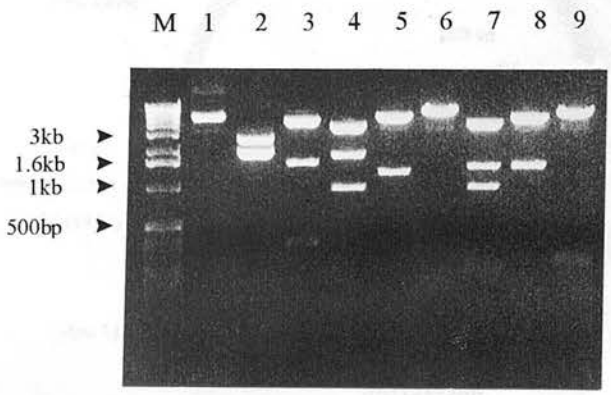


Fig. 4.9c. pSP65GCp53wtpuro maxi-prep. M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: SalI; Lane 6: EcoRV; Lane 7: PstI; Lane 8: PvuII; Lane 9: SphI.

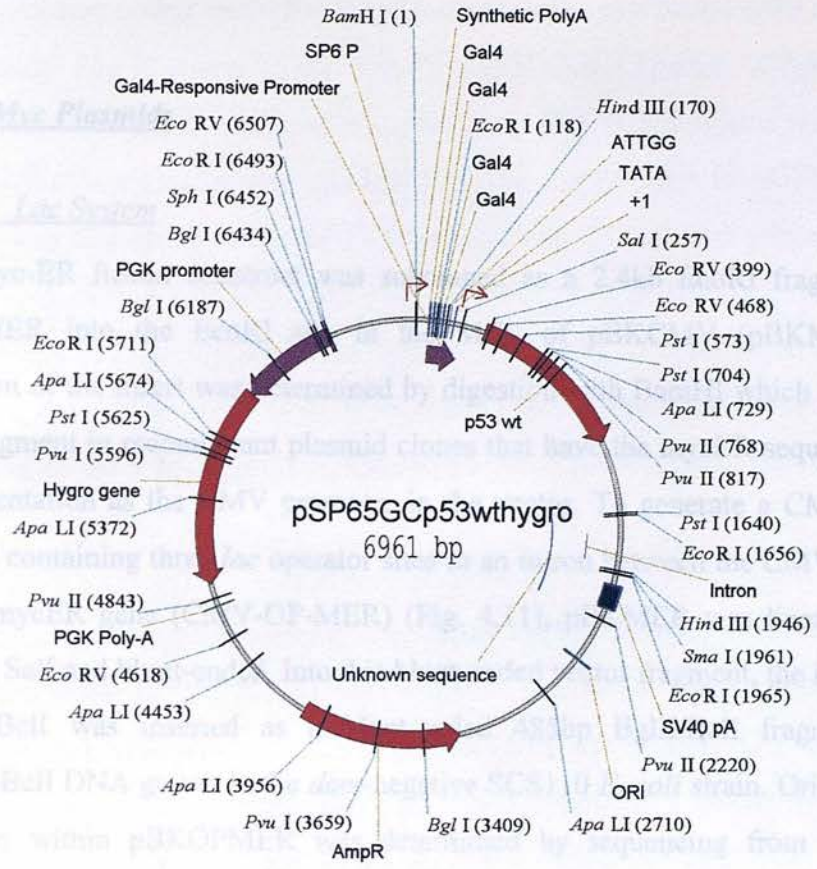


Fig. 4.10. pSP65GCp53wthygro and pSP65GCp53wtpuro.

4.2.1.3 Myc Plasmids

4.2.1.3.1 Lac System

The c-myc-ER fusion construct was subcloned as a 2.4kb EcoRI fragment from pMV7-MER into the EcoRI site in the MCS of pBKCMV (pBKMER). The orientation of the insert was determined by digestion with BamHI which produced a 1.4kb fragment in recombinant plasmid clones that have the mycER sequence in the same orientation as the CMV promoter in the vector. To generate a CMV-mycER construct containing three *lac* operator sites in an intron between the CMV promoter and the mycER gene (CMV-OP-MER) (Fig. 4.11), pBKMER was linearised with NheI and SalI and blunt-ended. Into this blunt-ended vector fragment, the intron from pOPI3N-BclI was inserted as a blunt-ended 485bp BglII/BclI fragment from pOP13N-BclI DNA grown in the *dam*-negative SCS110 *E. coli* strain. Orientation of the intron within pBKOPMER was determined by sequencing from the CMV promoter into the intron using the BKCMV primer (Stratagene). Two clones out of the six clones screened had the insert in the correct orientation. To generate a plasmid with the CMV-OP-MER construct which would also contain a puromycin resistance cassette, a 3.2kb NsiI/NotI fragment was subcloned as a blunt-ended fragment from pBKOPMER into blunt-ended, BstXI/NotI vector fragment from pOPRpuro (pCMVOPMER). Orientation of the insert was determined by digestion with HindIII (Figs. 4.12 to 4.14).

4.2.1.3.2 Tamoxifen-Inducible System

To isolate a wild-type c-*myc* cDNA, total RNA from the A549 human lung epithelial cell line was isolated, reverse transcribed and subjected to PCR with oligodeoxyribonucleotide primers designed to amplify a 1343bp fragment corresponding to bases 554-1879 of human c-Myc cDNA (GenEMBL accession number: v00568) which encompasses the ORF encoding c-MYC protein. The 5' primer (5987: myc5'; 5'- CC**GAATTC**GACGATGCCCCT-3', overlaps the c-Myc

translation initiation codon (underlined) and incorporates a synthetic EcoRI restriction half-site (Bold). The 3' primer (3212: myc3'; 5'-GGCCGCTTACGCACAAGAGTT-3', overlaps the translational stop codon (underlined). The PCR products were cloned into pGEM-T (Promega) at the equivalent of the EcoRV site in pGEM-5Zf(+). pGEMmyc was sequenced using the PCR primers and T7 and Sp6 primers and by subcloning each of two PstI fragments into the PstI site of pGEM-5Zf(+) and using the T7 and Sp6 primers. (pGEMmycS and pGEMmycL). A cytosine to adenosine mutation was detected at base 1440 of sequence v00568 which conserves the amino acid sequence of the c-MYC protein. The remainder of the sequence was identical to v00568.

To generate a Gal4-responsive expression vector (pSP65GCmyc) containing the c-myc cDNA, a single 1350bp EcoRI/NotI fragment pGEMmyc (blunt-ended with T4 DNA polymerase) was subcloned from into the EcoRV site of pSP65GC. Orientation of the *myc* gene was determined with PstI which produced a 784bp fragment. A puromycin resistance cassette was added to produce plasmid pSP65GCmycpuro by subcloning the 1.4kb PvuII/BamHI fragment (blunt-ended) from pPUR (de la Luna, 1988) into the SphI site (also blunt-ended) of pSP65GCmyc. The orientation of the puromycin resistance cassette was determined to be in the opposite direction to the Gal4-*myc* expression cassette by digestion with PstI which produced a 1774bp fragment (Fig. 4.15).

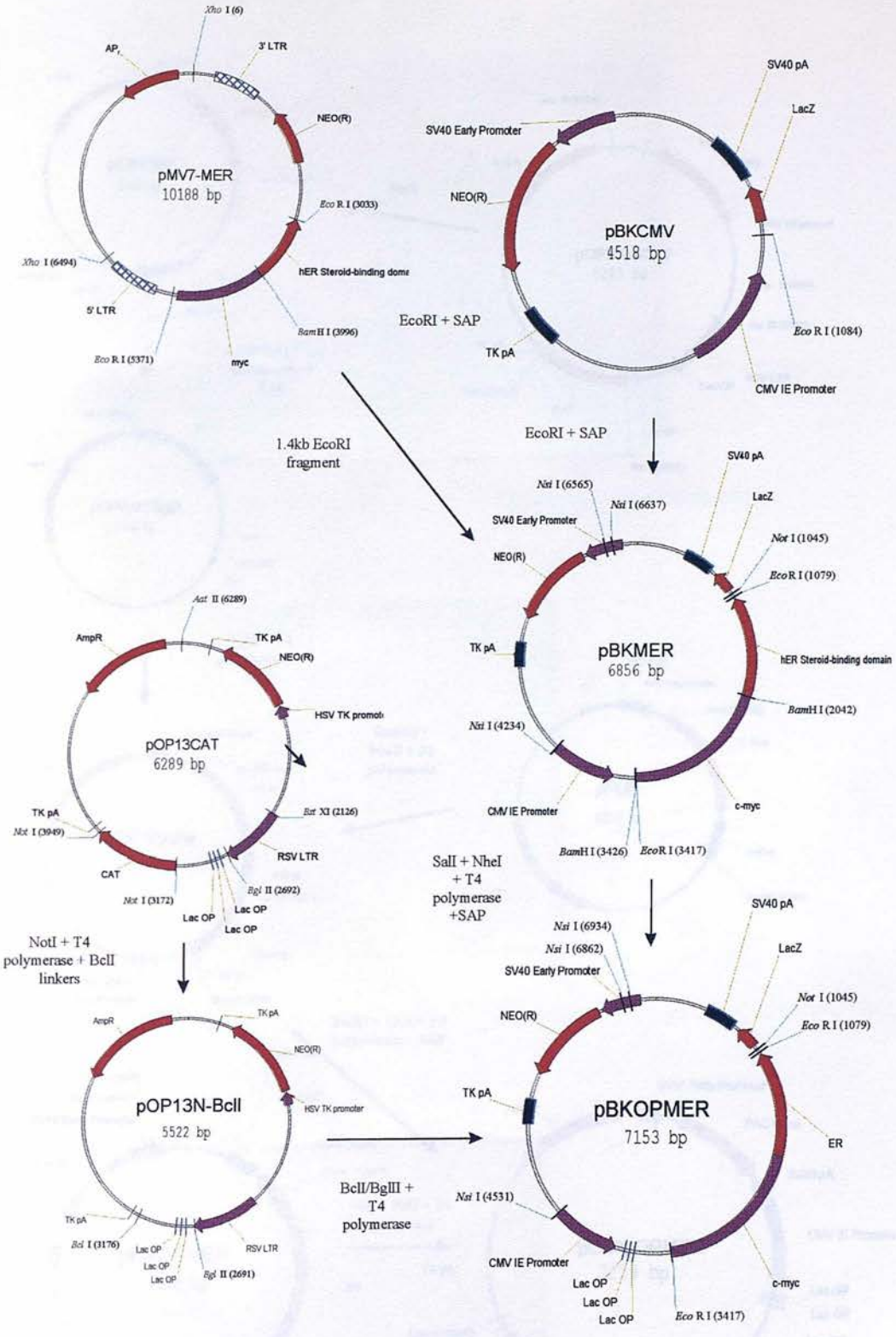


Fig. 4.11: Construction strategy for pBKOPMER. (Detailed maps for intermediates in Appendix C.)

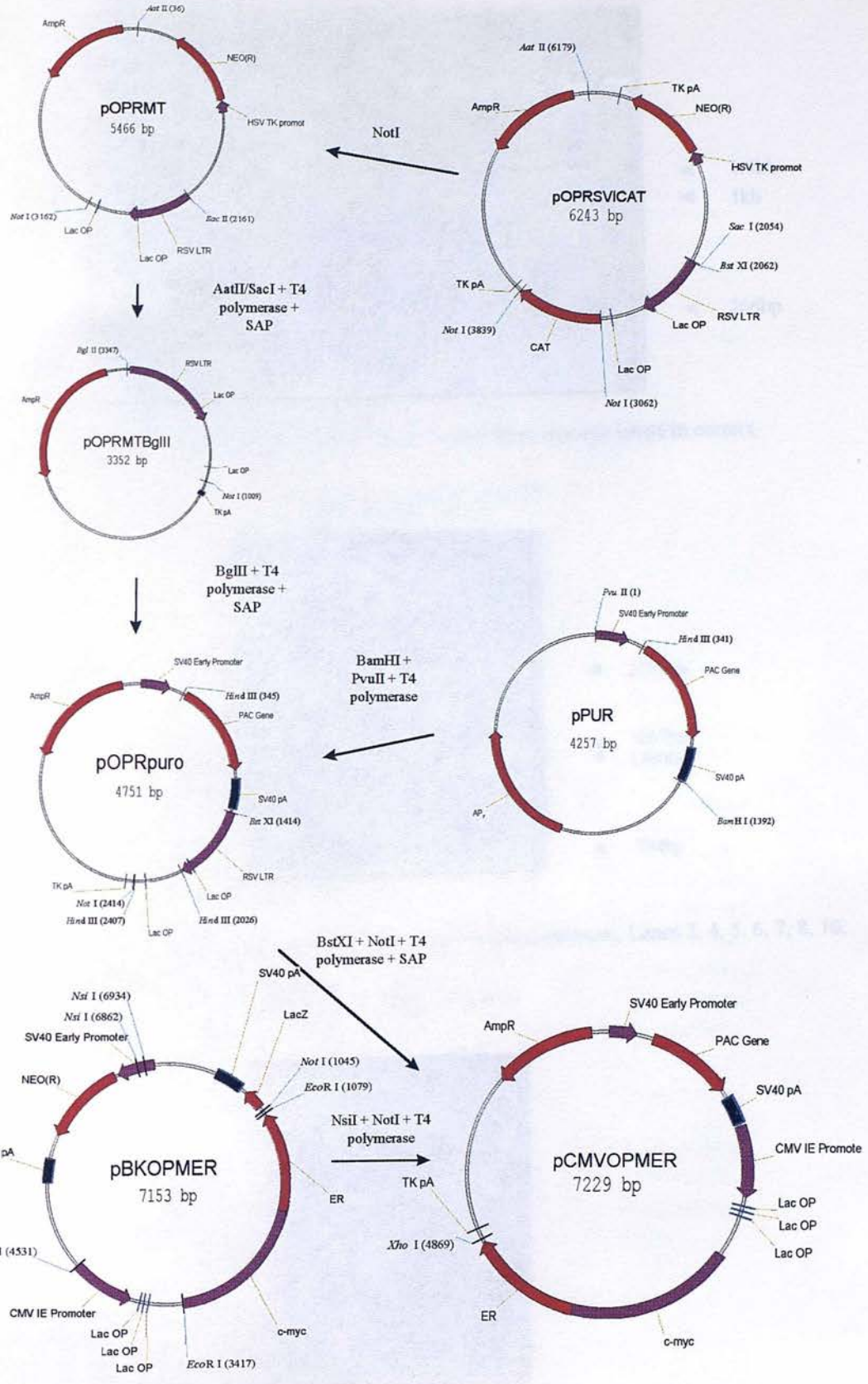


Fig. 4.12: Construction strategy for pCMVOPMER. (Detailed maps for intermediates in Appendix C.).

Fig. 4.13a. pOPRMER mini-preps, BamHI. Lanes 3 and 7 have *myc*-ER insert in correct orientation.

Fig. 4.13b. pCMVOPMER minipreps, HindIII. Lane 1 nonrecombinant, Lanes 2, 4, 5, 6, 7, 8, 10, 11, 12 correct orientation.

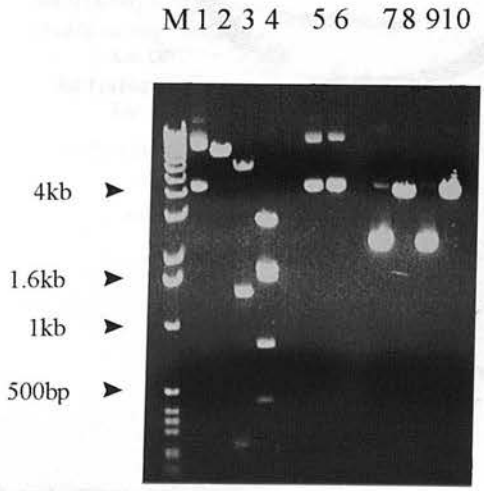
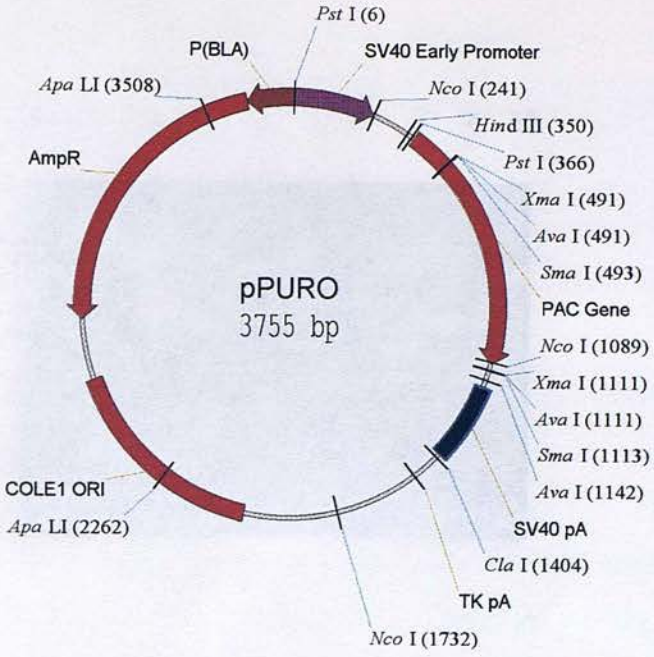


Fig.4.13c. pCMVOPMER (lanes 1 to 6) **and pPURO** (lanes 7 to 10) **maxi-preps:** M: Life Tech. 1kb ladder. Lane 1: uncut DNA; Lane 2: XhoI; Lane 3: BamHI: Lane 4: HindIII; Lane 5: BstXI: Lane 6: NotI; Lane 7: uncut pPURO DNA; Lane 8: HindIII; Lane 9: BstXI; Lane 10: NotI.



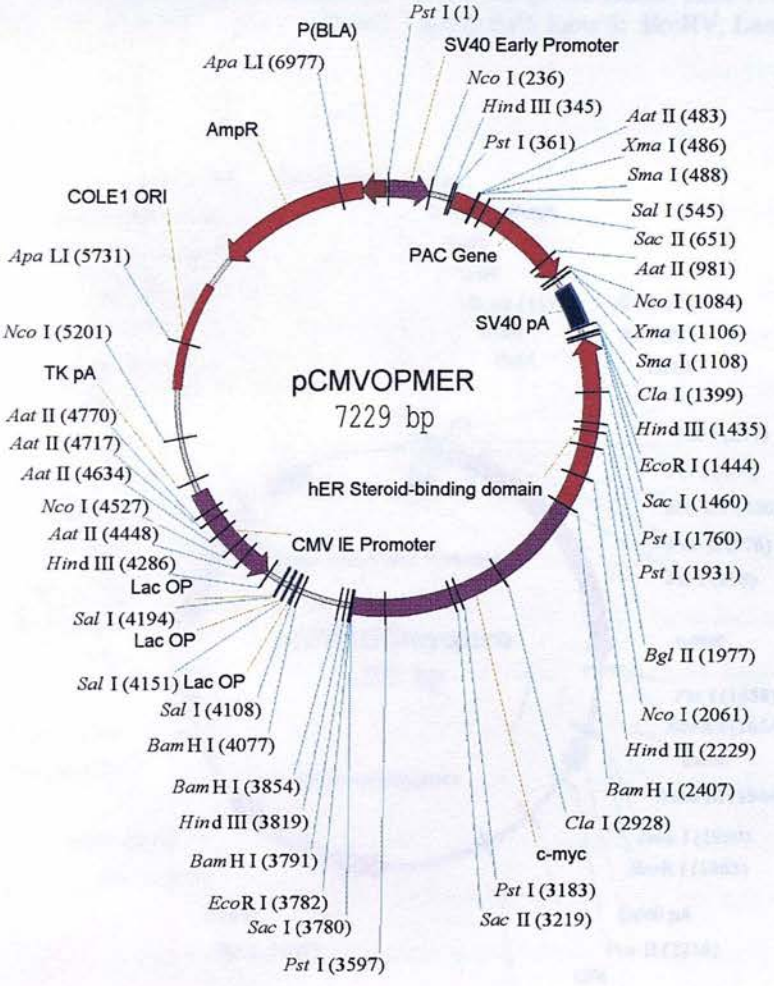
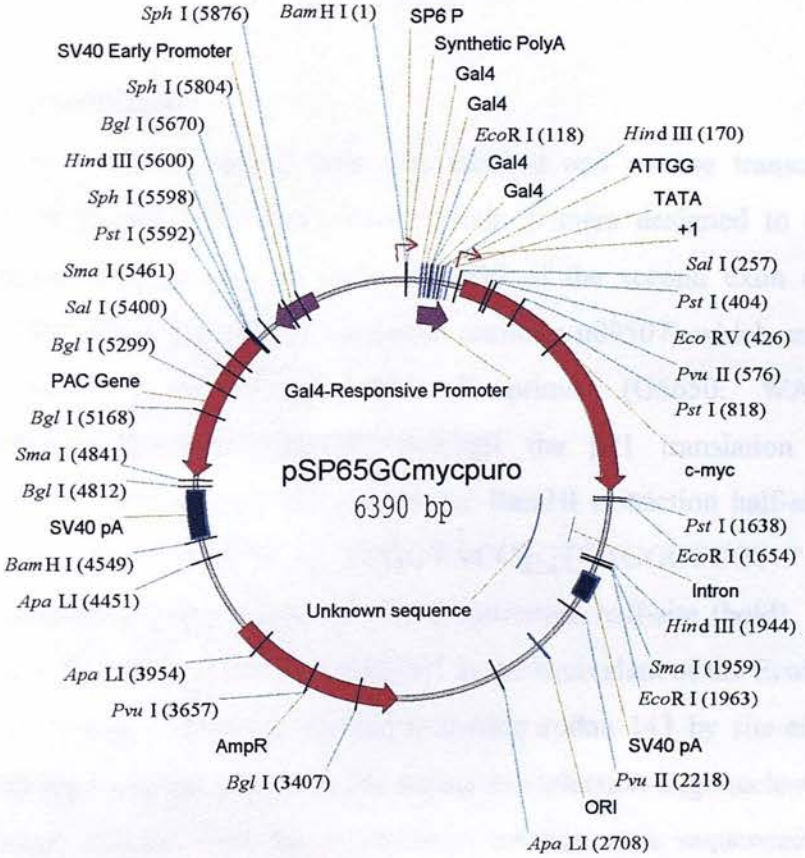


Fig. 4.14. pPURO and pCMVOPMER.

Fig. 4.15. pSP65GCmycpuro. Maxi-prep (above): M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: SalI; Lane 6: EcoRV; Lane 7: PstI; Lane 8: PvuII.



4.2.1.4 Plasmids containing p21$^{Waf1/Cip1}$

4.2.1.4.1 Full-length p21.

The open reading frame from the 2nd and 3rd exons (amino acids 1 to 159 and stop codon (TGA) of murine p21 WAF1/CIP1 (u09507: bases 201-639) was amplified by high fidelity RTPCR from 129/Sv/ola total murine liver RNA using primers WAF5' (G5650: 5'-CA**GGATCC**ATGCCAATCCTGGT-3'; which overlaps the p21 translation initiation codon (underlined) and incorporates a synthetic BamHI restriction half-site (bold)) and WAF23 (15027: 5'-GGCACTTCAGGGTTTTCTCTTGC-3' ; and includes the stop codon (codon 160: underlined) (Fig. 4.16a). The PCR products were cloned as a 496bp fragment into pGEM-T at the equivalent of the EcoRV site in pGEM5Zf(+) (Promega). The resulting plasmid, pGEMWAF1-160 (Fig 4.16b), was sequenced using the PCR primers, primer WAF 5'Q (2645), and the T7 promoter primer which bind within the vector. No alterations from the published p21 sequence were found

4.2.1.4.2 Truncated p21.

Total RNA from 129/Sv mouse liver was isolated and reverse transcribed and subjected to PCR with oligodeoxyribonucleotide primers designed to amplify a 446bp fragment corresponding to bases 200-639 of the second exon of murine p21WAF1/CIP1 cDNA (GenEMBL accession number: u09507) which encodes the first 143 amino acids of p21. The 5' primer (G5650: WAF5'), 5'-CA**GGATCC**ATGCCAATCCTGGT-3', overlaps the p21 translation initiation codon (underlined) and incorporates a synthetic BamHI restriction half-site (bold). The 3' primer (G5649: WAF3'), 5'- TC**GGTACC**TGTCAGGCTGGT-3', contains codon 143 (underlined) and a synthetic KpnI restriction half-site (bold). The PCR products (Fig 4.16a) were cloned into pGEM-T at the equivalent of the EcoRV site in pGEM5Zf(+). A stop codon was inserted following codon 143 by site-elimination (KpnI) site-directed mutagenesis using the mutagenic/selection oligonucleotide W26. Eight individual colonies from the mutagenesis reaction were sequenced with the

primer WAF 5'Q and all (100%) were found to contain the inserted stop codon (TGA) at codon position 143 (pGEMWAF1-143) (Fig. 4.16c). The rest of the *waf-1* reading frame was sequenced using the PCR primers and the T7 and Sp6 promoter primers. A conservative mutation (T to G) was found at base 581 (numbered according to u09507) of the p21 sequence.

4.2.1.4.3 Lac system

A 512bp BamHI/NotI fragment was subcloned from pGEMWAF1-160 (blunt-ended with T4 DNA polymerase) into the (blunt) NotI site of pOPRMT to generate plasmid pOPRWAF1-160. Orientation of the insert was confirmed by EcoRI/SmaI (901bp) digest. All fusion sites were destroyed in the cloning process (Fig. 4.17).

pGEMWAF1-143 was digested with BamHI, blunt-ended and ligated to 10mer NotI linkers. The resulting 520bp NotI fragment was then subcloned into the NotI site of pOPRMT. In a recombinant clone that was designated pOPRWAF1-143, the 5' NotI site was destroyed (Appendix C.).

4.2.1.4.4 Tamoxifen-inducible system.

The 512bp BamHI/NotI fragment from pGEMWAF1-160 (blunt-ended with T4 DNA polymerase) was subcloned into the EcoRV site of pSP65GCpuro to generate pSP65GCWAF1-160puro. Orientation of the insert was confirmed by digestion with PstI (1350bp) (Figs. 4.18a and 4.19).

To generate plasmid pSP65GCWAF1-143puro, a 464bp BamHI/NotI fragment (blunt-ended with T4 DNA polymerase) was subcloned from pGEMWAF1-143 into the EcoRV site of pSP65GCpuro. Orientation of the insert was verified using PstI (1350bp) (Figs. 4.18b and 4.19).

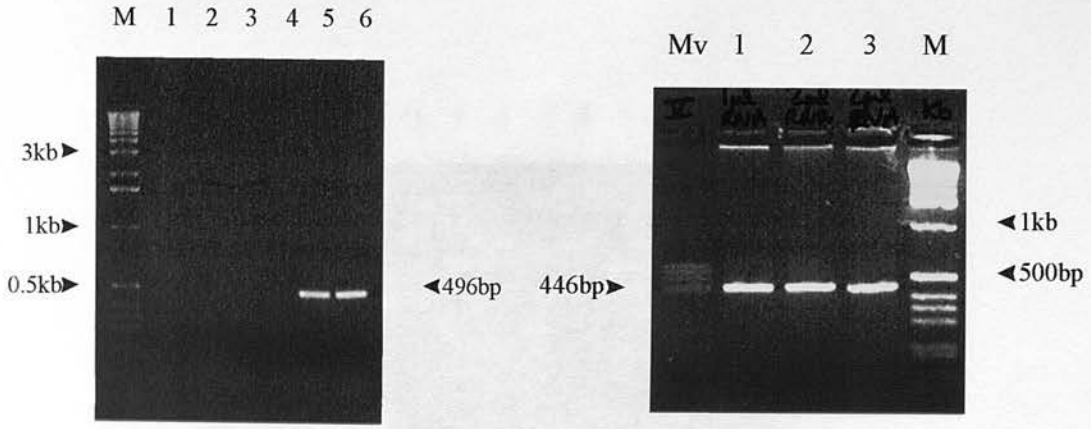


Fig. 4.16a. Left: p21WAF (Full-length: codons 1-160) Hi-fidelity RT-PCR, M; Life Tech. 1kb ladder. Lane 1: 0mM $MgCl_2$; Lane 2: 0.5mM; Lane 3: 0.75mM; Lane 4: 1.00mM; Lane 5 1.25mM; Lane 6: 1.5mM. **Right: p21WAF (Truncated: codons 1-143) RTPCR** Mv: Boehringer Mannheim Marker V. Lane 1: 1 µl mouse liver RNA. Lane 2: 2µl RNA (-DMSO), Lane 3: 2µl RNA (+DMSO).

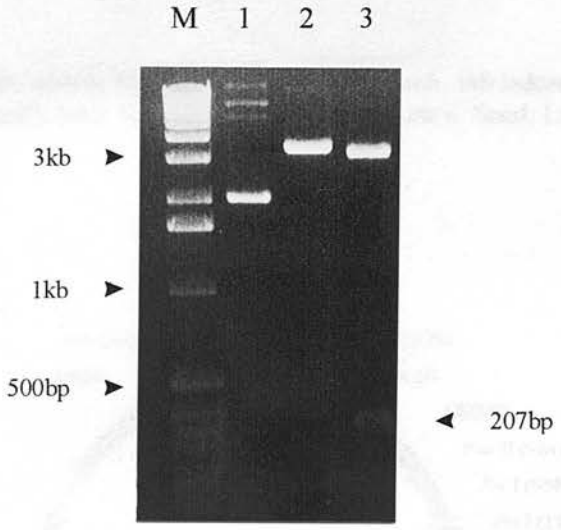


Fig. 4.16b. pGEMWAF1-160 maxi-prep. M; Life Tech. 1kb ladder. Lane 1; uncut plasmid; Lane: 2: BamHI; Lane 3: PstI.

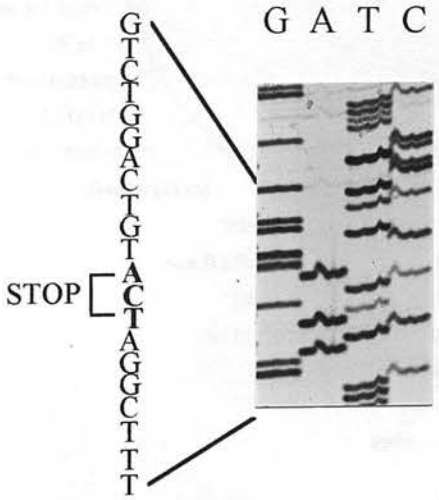


Fig. 4.16c. Sequencing of Stop codon inserted by site-directed mutagenesis into pGEMWAF1-143.

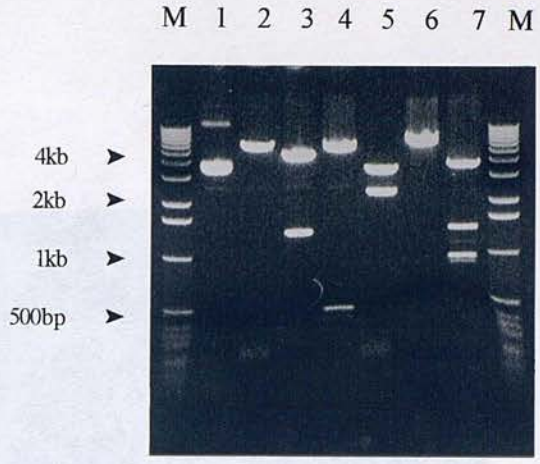
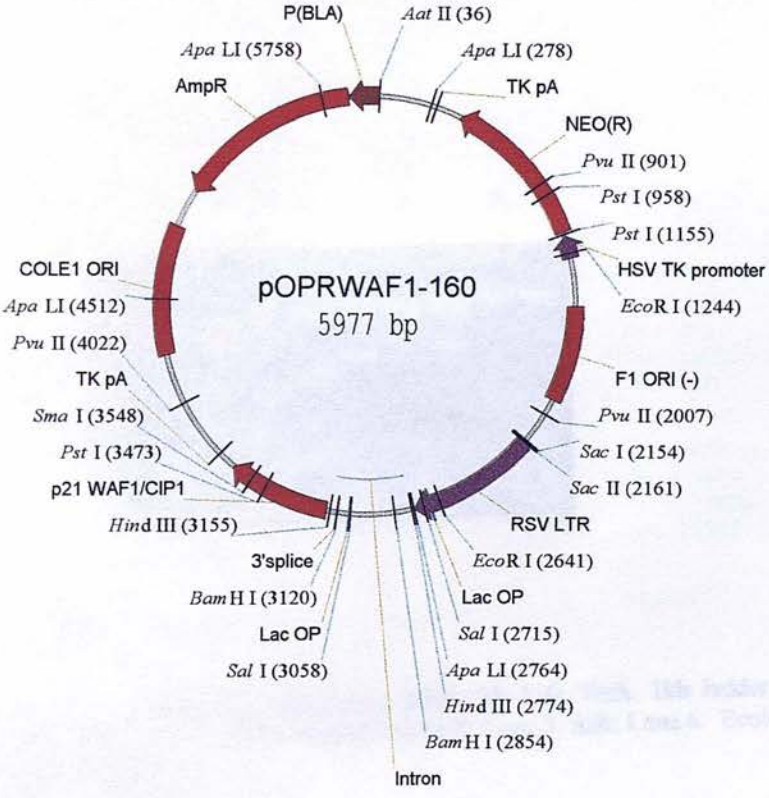


Fig. 4.17. pOPRWAF1-160. Above: Maxiprep DNA. M; Life Tech. 1kb ladder. Lane 1: uncut DNA; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: PstI; Lane 6: SmaI; Lane 7: EcoRI/SmaI.



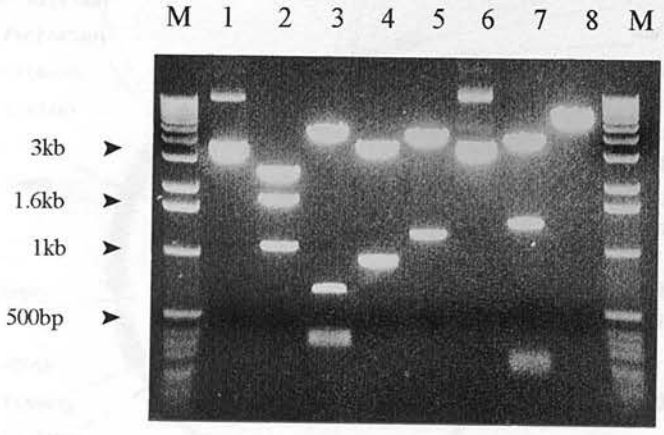


Fig. 4.18a. pSP65GCWAF1-160puro maxi-prep DNA. M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: SalI; Lane 6: EcoRV; Lane 7: PstI; Lane 8: PvuII.

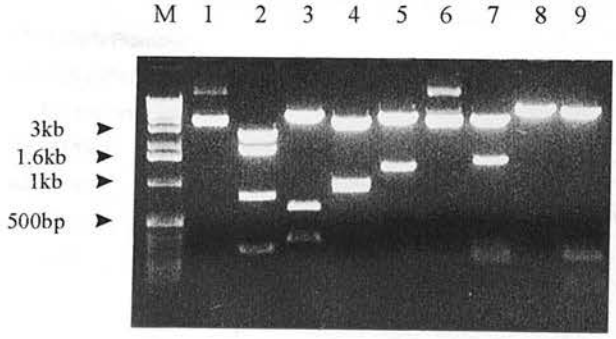


Fig. 4.18b. pSP65GCWAF1-143puro maxi-prep DNA. M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: SalI; Lane 6: EcoRV; Lane 7: PstI; Lane 8: PvuII. Lane 9: SphI.

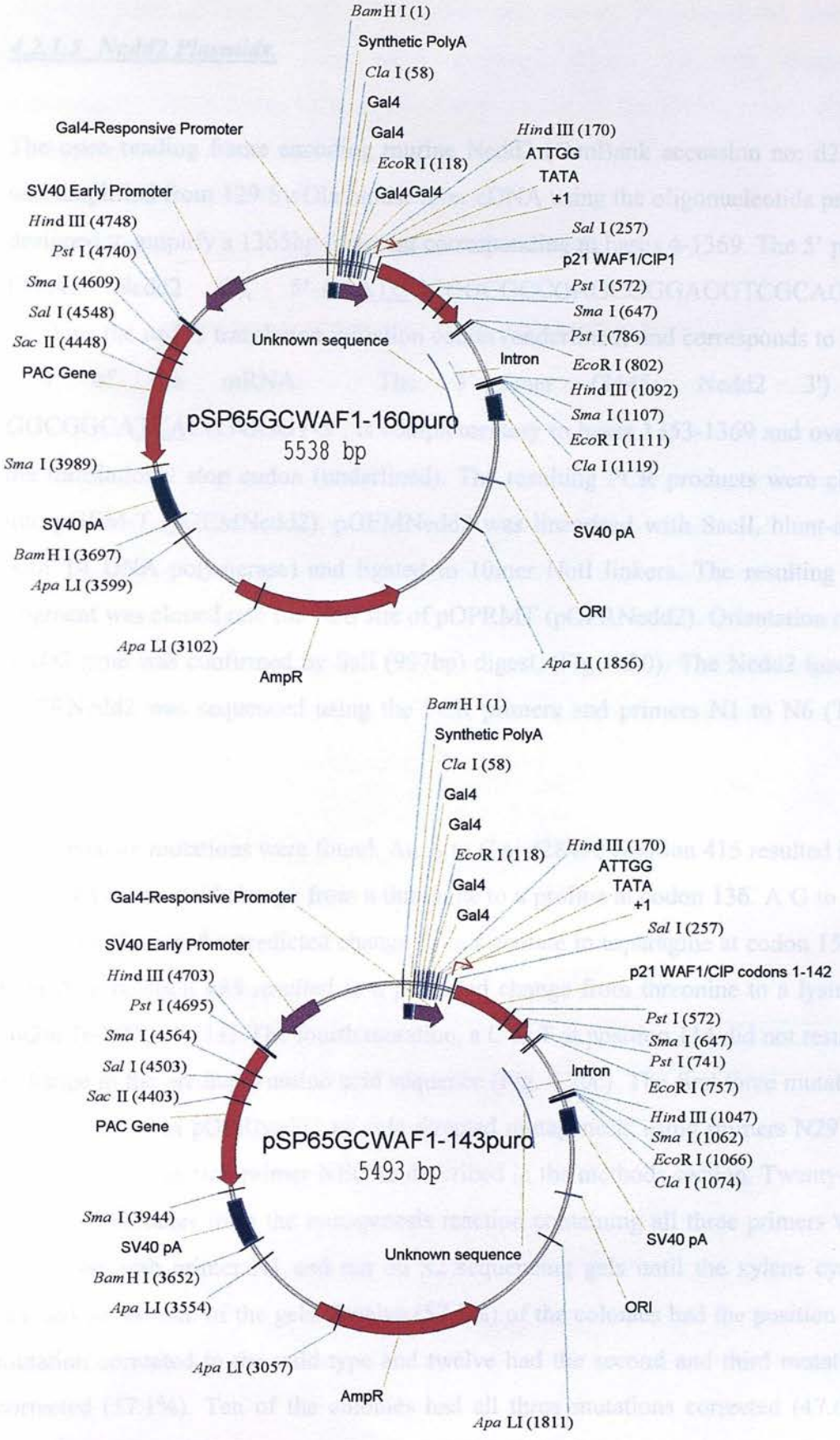


Fig. 4.19. pSP65GCWAF1-160puro and pSP65GCWAF1-143puro.

4.2.1.5 Nedd2 Plasmids.

The open reading frame encoding murine Nedd2 (GenBank accession no: d28492) was amplified from 129/Sv/Ola mouse liver cDNA using the oligonucleotide primers designed to amplify a 1365bp fragment corresponding to bases 4-1369. The 5' primer (3444: Nedd2 5'), 5'-AAATGGCGGCGCCGAGCGGGAGGTCGCAGT-3', overlaps the nedd2 translation initiation codon (underlined) and corresponds to bases 5-34 of the mRNA. The 3'-primer (3445: Nedd2 3') 5'-GGCGGCATCACGTGGGT-3', is complementary to bases 1353-1369 and overlaps the translational stop codon (underlined). The resulting PCR products were cloned into pGEM-T (pGEMNedd2). pGEMNedd2 was linearised with SacII, blunt-ended with T4 DNA polymerase) and ligated to 10mer NotI linkers. The resulting NotI fragment was cloned into the NotI site of pOPRMT (pOPRNedd2). Orientation of the *Nedd2* gene was confirmed by SalI (997bp) digest. (Fig. 4.20). The Nedd2 insert in pOPRNedd2 was sequenced using the PCR primers and primers N1 to N6 (Table 4.2).

Four separate mutations were found. An A to C at d28492 position 415 resulted in an predicted amino acid change from a threonine to a proline at codon 136. A G to A at position 478 caused a predicted change from aspartate to asparagine at codon 158. A C to A at position 485 resulted in a predicted change from threonine to a lysine at codon 160 (Fig. 4.21a). The fourth mutation, a C to T at position 114 did not result in a change in the predicted amino acid sequence (Fig. 4.20c). The first three mutations were corrected in pOPRNedd2 by side directed mutagenesis using primers N29 and N30 with the selection primer NEO as described in the methods section. Twenty-one individual colonies from the mutagenesis reaction containing all three primers were sequenced with primer N1 and ran on S2 sequencing gels until the xylene cyanol reached the bottom of the gels. Twelve (57.1%) of the colonies had the position 136 mutation corrected to the wild-type and twelve had the second and third mutations corrected (57.1%). Ten of the colonies had all three mutations corrected (47.6%). One clone (clone 10) was selected and maxiprep DNA from clone 10 was sequenced

using the panel of sequencing primers described above. No deviations from the published wild-type sequence were detected. Clone 10 was designated pOPRNedd2(10). In order to sequence the 5' region of the Nedd2 insert, plasmid pOPRNedd2(10) was treated with BamHI, recircularised (pOPRNedd2BamHI) and sequenced using the BKRSV primer (Stratagene) which binds to a region at the 3' end of the RSV LTR. Again, no deviations from the wild type sequence were detected.

By subcloning a blunt-ended NotI fragment from pOPRNedd2(10) into the EcoRV site of pSP65GC, the plasmid pSP65GCNedd2 was generated. The orientation of the Nedd2 gene was checked with SalI which produced a 1008bp diagnostic fragment length. The puromycin resistance cassette from pPUR (Clontech) (de la Luna, 1988) was subcloned as a 1392bp PvuII/BamHI blunt-ended fragment into the SphI site of pSP65GCNedd2 (Figs. 4.21b and 4.22). Recombinant plasmids contained the puromycin resistance cassette in the same orientation as the Gal4-*Nedd2* cassette (as confirmed by an HindIII digest which produced a 1671bp fragment) and were designated pSP65GCNedd2puroSD (Fig. 4.22).

Table 4.2. Nedd2 sequencing primers.

Primer	Sequence	Nedd2 position (bp)
N1	5'-GGCAGTTTCAGCCAGAATGT-3'	241-260
N2	5'-TCCCTTTCTCGGTGTGTGA-3'	422-450
N3	5'-TTGGCACTGGTGCTGAGCAAT-3'	604-624
N4	5'-TTGCACAGTTACCTGCACAC-3'	776-795
N5	5'-AGCATGTCGTGGAGATGAGA-3'	957-976
N6	5'-GCCTTAAAGGTAATGCTGC-3'	1108-1127

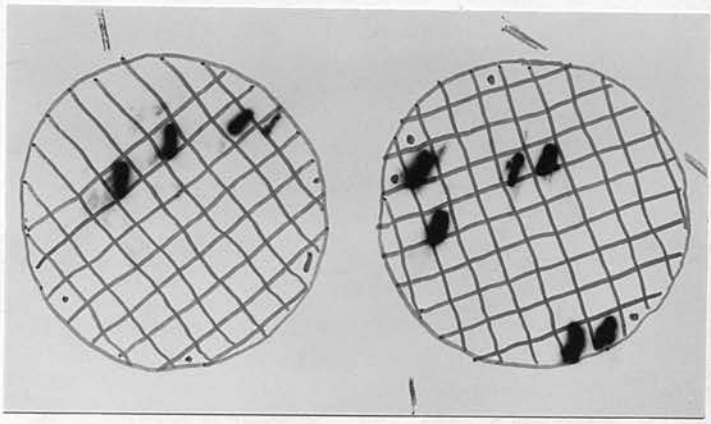


Fig. 4.20a. Colony hybridisation screen for pOPRNedd2; Nedd2 cDNA probe.

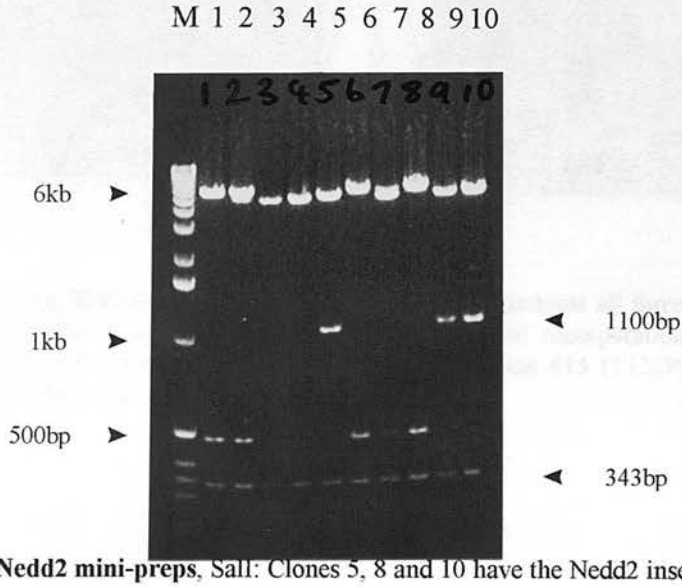


Fig. 4.20b. pOPRNedd2 mini-preps, SalI: Clones 5, 8 and 10 have the Nedd2 insert in the correct orientation.

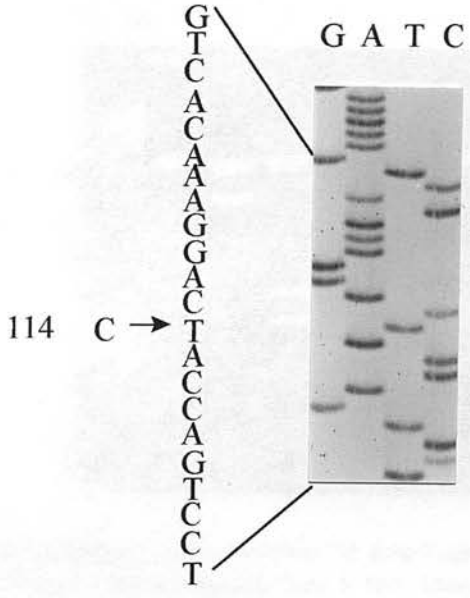


Fig. 4.20c. Sequencing of pOPRNedd2, 5' Nedd2 primer. The C to T change at position 114 is conservative

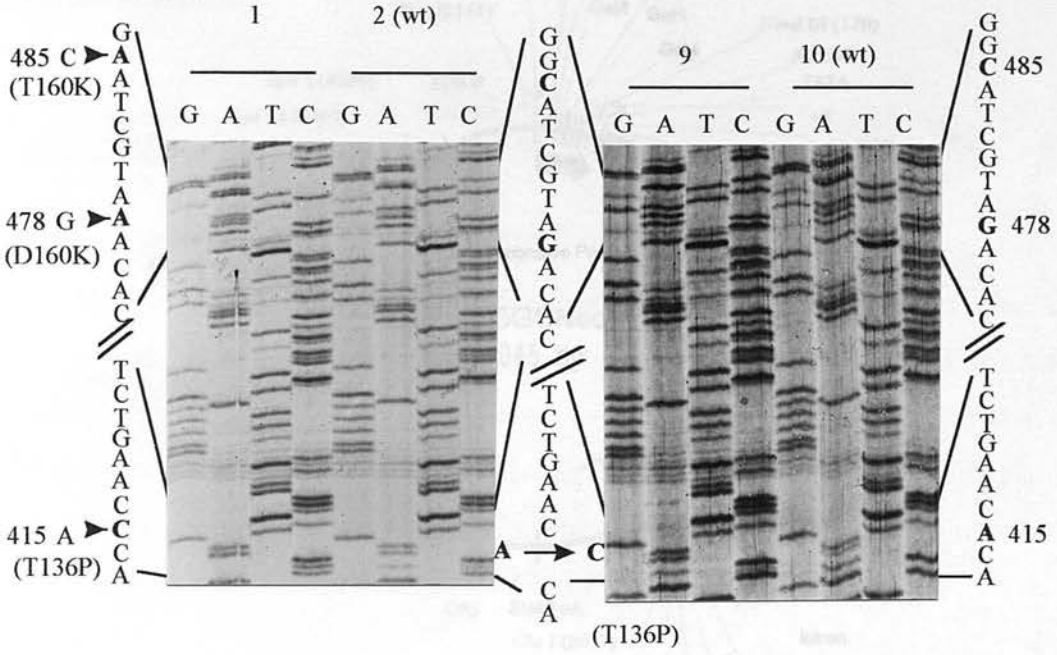


Fig. 4.21a. Correcting Mutagenesis of pOPRNedd2. Clone 1 contains all three mutations that alter the protein sequence. Clones 2 and 10 are wild-type as a result of incorporation of both the correcting mutagenic oligonucleotides. Clone 9 retains a mutation at position 415 (T136P) and represents a clone that has incorporated only one primer.

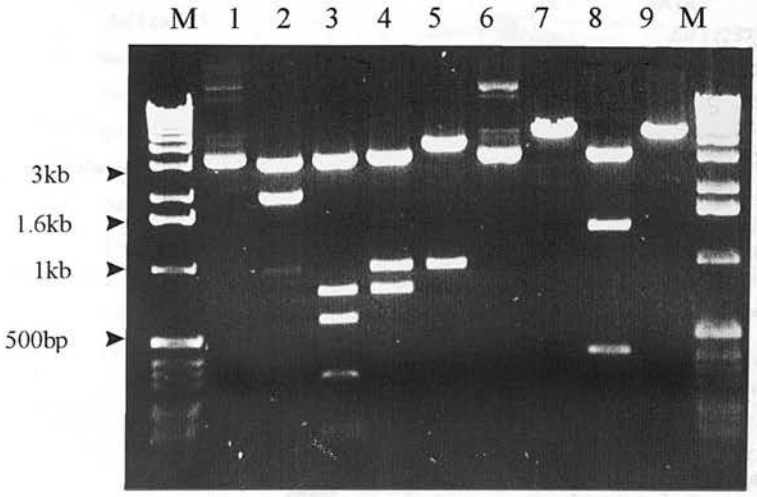


Fig. 4.21b. pSP65GCNedd2 Maxi-prep DNA. M: Life Tech. 1kb ladder. Lane 1: uncut plasmid; Lane 2: BamHI; Lane 3: EcoRI; Lane 4: HindIII; Lane 5: SalI; Lane 6: EcoRV; Lane 7: PstI; Lane 8: PvuII; Lane 9: SphI.

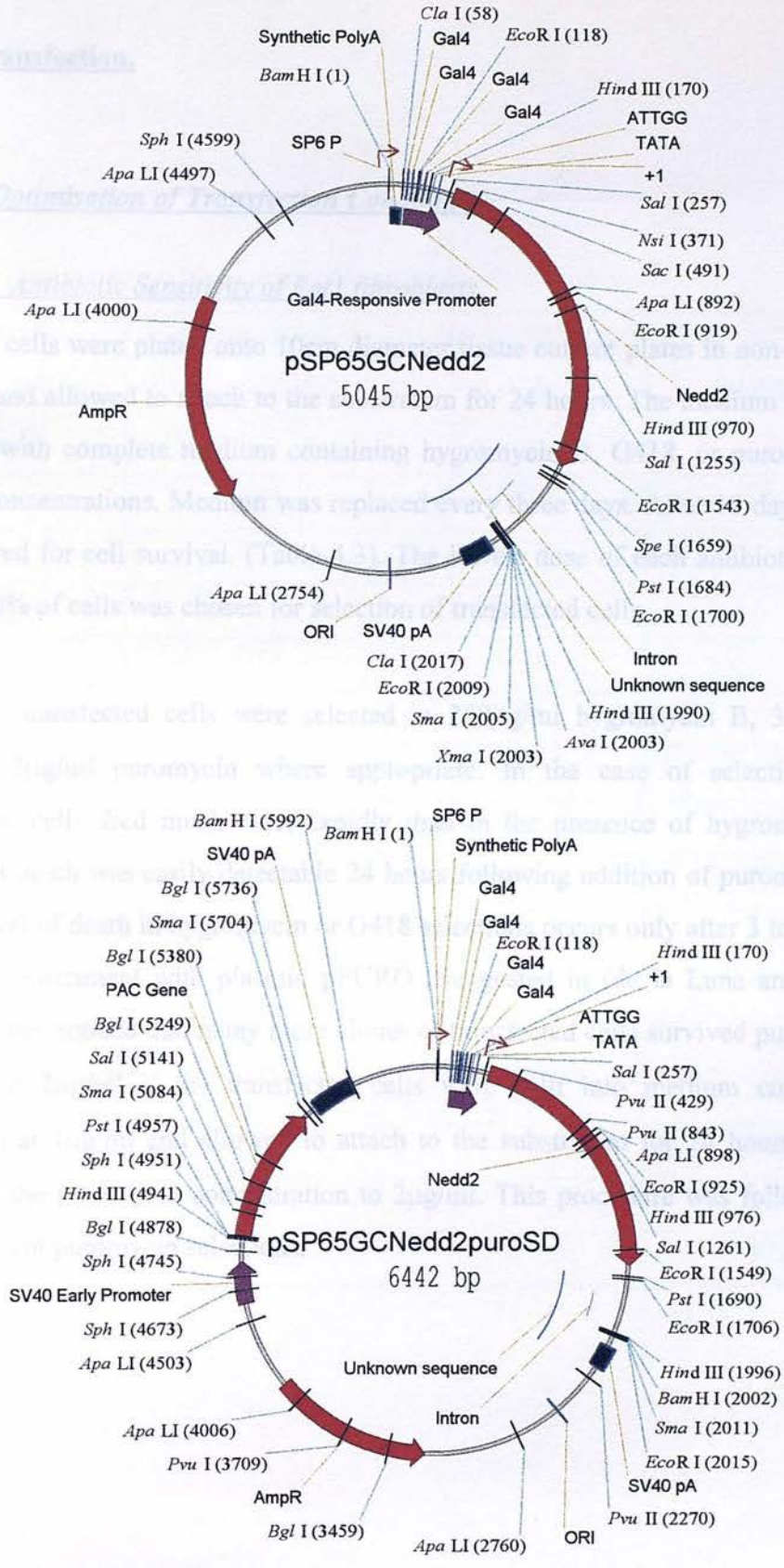


Fig. 4.22. pSP65GCNedd2 and pSP65GCNedd2puroSD.

4.2.2 Transfection.

4.2.2.1 Optimisation of Transfection Conditions

4.2.2.1.1 Antibiotic Sensitivity of Rat1 fibroblasts.

0.5×10^6 cells were plated onto 10cm diameter tissue culture plates in non-selective medium and allowed to attach to the substratum for 24 hours. The medium was then replaced with complete medium containing hygromycin B, G418, or puromycin at various concentrations. Medium was replaced every three days. After 10 days, plates were scored for cell survival. (Table 4.3). The lowest dose of each antibiotic which killed 100% of cells was chosen for selection of transfected cells.

Therefore, transfected cells were selected in 250μg/ml hygromycin B, 350μg/ml G418 or 2μg/ml puromycin where appropriate. In the case of selection with puromycin, cells died much more rapidly than in the presence of hygromycin or G418, cell death was easily detectable 24 hours following addition of puromycin. A similar level of death in hygromycin or G418 selections occurs only after 3 to 4 days. In a pilot experiment with plasmid pPURO (Suggested in (de la Luna and Ortin, 1992)), it was noticed that many more clones of transfected cells survived puromycin selection at 2μg/ml if the transfected cells were split into medium containing puromycin at 1μg/ml and allowed to attach to the substratum for 24 hours before increasing the puromycin concentration to 2μg/ml. This procedure was followed in all subsequent puromycin selections.

Table 4.3. Determination of Antibiotic Concentrations for Selection.

Antibiotic Concentration. (μg/ml)	Cells Surviving in Hygromycin B.	Cells Surviving in G418.	Cells Surviving in puromycin.
0	+++	+++	+++
1	N/D	N/D	+
2	N/D	N/D	-
3	N/D	N/D	-
4	N/D	N/D	-
5	N/D	N/D	-
50	++	++	N/D
100	++	++	N/D
150	+	++	N/D
200	+	++	N/D
250	-	+	N/D
300	-	+	N/D
350	-	-	N/D
400	-	-	N/D
500	-	-	N/D

4.2.2.1.2 Determination of Optimal pH of 2xBBS.

The optimal conditions for transfection of DNA into monolayer cultures of mammalian cells by the calcium phosphate coprecipitation method as modified by Chen and Okayama (Chen and Okayama, 1987) lie within narrow limits. Transfection efficiency is affected by a number of factors including the pH of the 2 x BBS solution, the initial pH of the medium, the age and concentration of $CaCl_2$ and DNA preparations and the CO_2 concentration in the incubator. If the overall pH of the medium is too acidic following addition of the BBS/$CaCl_2$/DNA mixture, very little precipitate forms. Alternatively if it is too alkaline the precipitate clumps together rapidly following addition to the medium. Consequently, parallel batches of 2xBBS solution were made up and the pH was adjusted close to pH 6.95 which was judged to be optimal in the experiments of Chen and Okayama. Transfections of plasmid pPURO were thus carried out with 20μg of DNA and 2xBBS solutions ranging from pH 6.95 to pH 6.98. Cells were split 1 in 10, 24 hours after transfection into replicate 10cm culture plates and selected in puromycin. Surviving colonies were fixed, stained with Giemsa and counted after 10 days. The optimal batch of 2 x BBS was that adjusted to pH 6.97. (Figs. 4.23 and 4.24) This pattern was confirmed in two independent experiments.

4.2.2.1.3 Determination of Optimal Plasmid DNA Concentration.

The quality of the calcium phosphate / DNA precipitate that develops during the overnight incubation step is markedly affected by the amount of plasmid DNA used. Too little DNA results in a coarse, flocculent precipitate. Too much DNA results in a very fine precipitate. The optimal DNA concentration results in an even, granular precipitate that is visible at x100 magnification (Chen and Okayama, 1987). In order to determine the optimal DNA concentration for pPURO, 10, 15, 20, or 25 μg of DNA was transfected using the optimal 2 x BBS buffer (pH6.97). Fig. 4.26 shows the morphology of the precipitates. The photograph of cells transfected with 20 μg of pPURO show an even granular precipitate that most resembles that of Chen and Okayama (Chen and Okayama, 1987). At this DNA concentration, the transfection

efficiency (as measured by average colonies per plate) was the highest by at least 2 to 3 fold (Figs 4.25 and 4.27). This pattern was also found in two independent experiments.

From these data it can be concluded that DNA concentration has a much more marked effect upon the transfection efficiency in Rat1 cells than small changes in the pH of the 2x BBS buffer. Therefore, similar experiments were carried out for other expression constructs to determine whether there was any variation in the optimal amount of plasmid DNA from construct to construct. As it was expected that some of the expression vectors might inhibit colony formation, it was assumed that the quality of the precipitate was related to the transfection efficiency. Table 4.4 details the concentration of expression plasmids that produced precipitates with an even, granular appearance.

The optimal plasmid concentration could not be predicted from the size of the plasmid, nor from the series of vectors to which any particular plasmid belonged. It may be that variations in the quality or age of the plasmid preparations may affect the quality of the precipitate. However, all plasmids used for transfection were prepared using Qiagen columns, were less than 6 months old and had $OD_{260/280}$ ratios of between 1.70 and 1.80. Alternatively, pipetting or DNA quantitation errors may account for variations in optimal DNA concentration between plasmids.

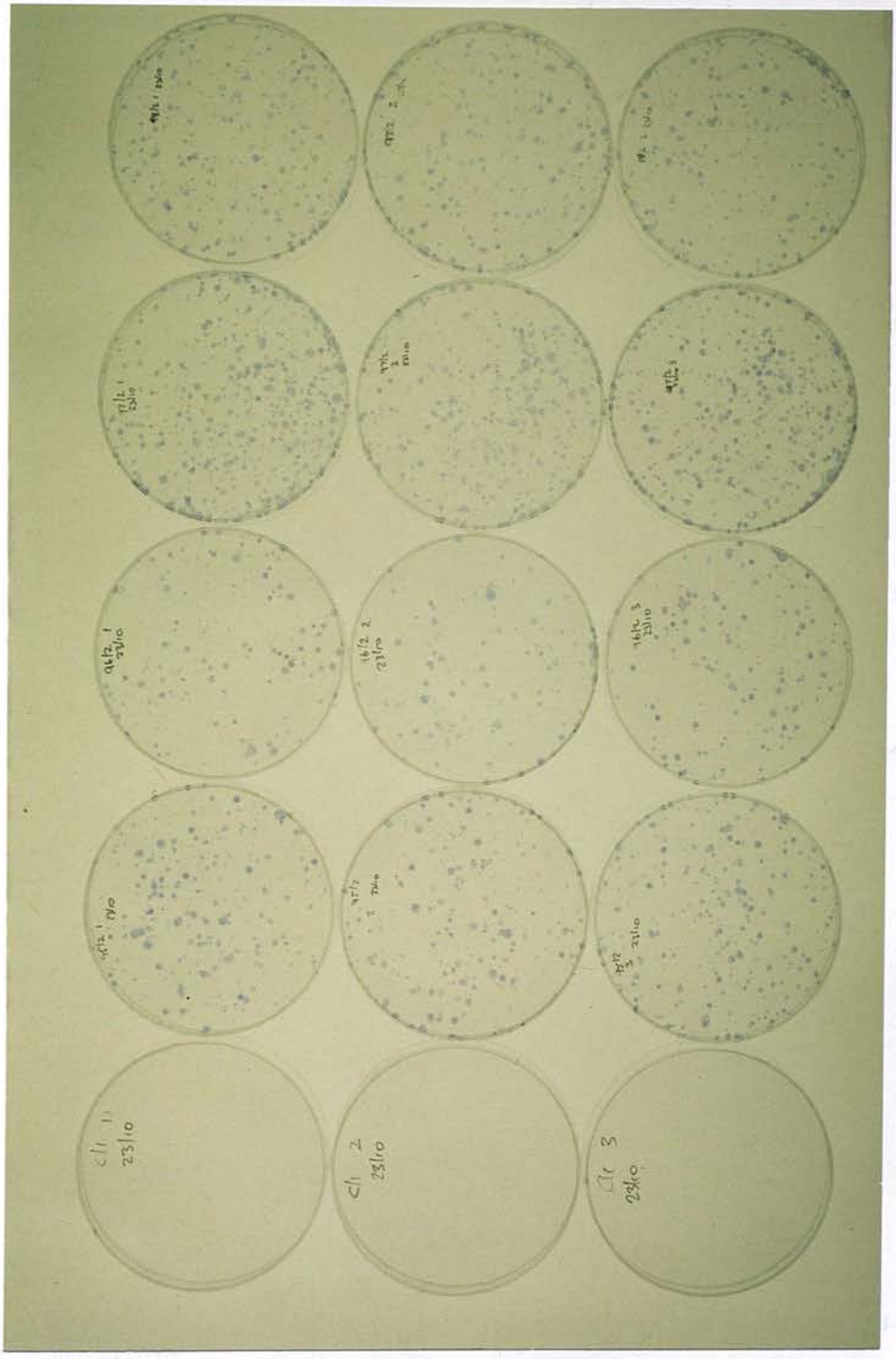


Fig. 4.23: Optimisation of calcium phosphate-mediated transfection (Chen and Okayama, 1987): 2xBBS pH: Colonies stained with Giemsa following transfection with pPURO and selection in 2μg/ml puromycin. (Bottom to Top Rows) Control transfection with non-relevant selectable marker (pBKCMV); Transfections with pPURO: pH 6.95; pH 6.96; pH6.97; pH 6.98.

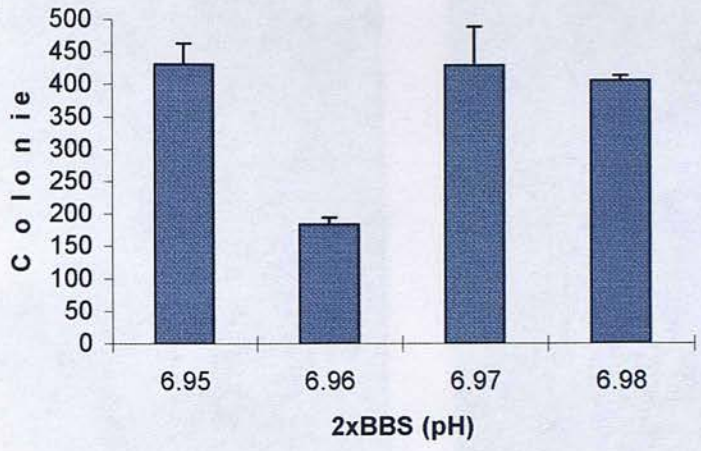


Fig. 4.24. Mean number of colonies per plate (+SEM) following transfection of pPURO in a 2xBBS buffer batch testing experiment.

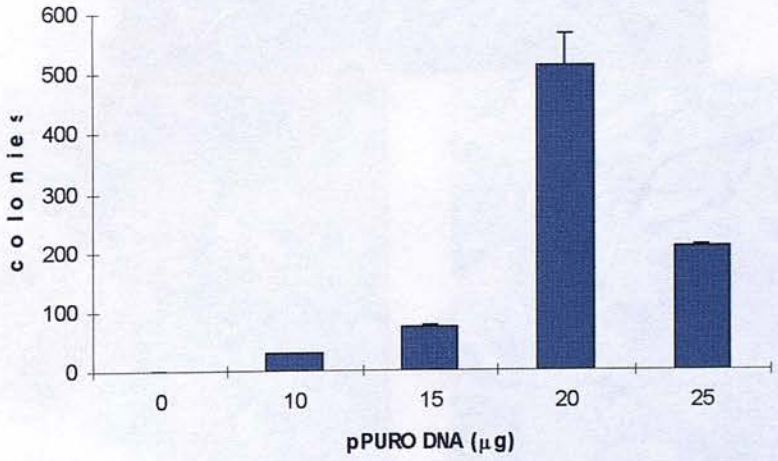


Fig. 4.25. Mean numbers of colonies per plate (+SEM) following transfection with pPURO with various amounts of DNA.

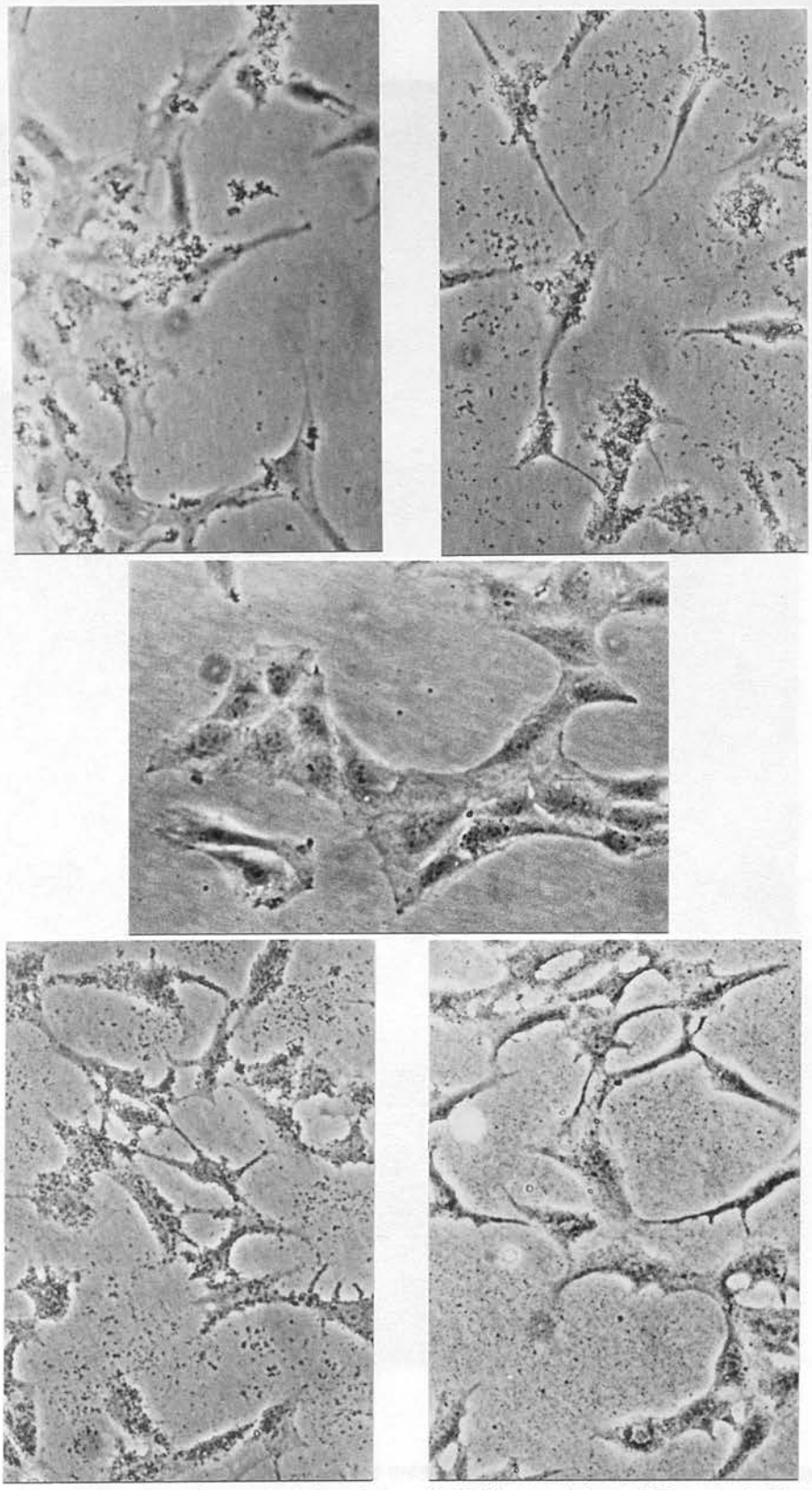


Fig. 4.26: Appearance of calcium phosphate/plasmid DNA precipitate (Chen and Okayama method) on Rat1 cells following overnight incubation. Top left: 10μg pPURO; Top right: 15μg; Centre: untransfected cells. Bottom left: 20μg; Bottom right: 25μg.

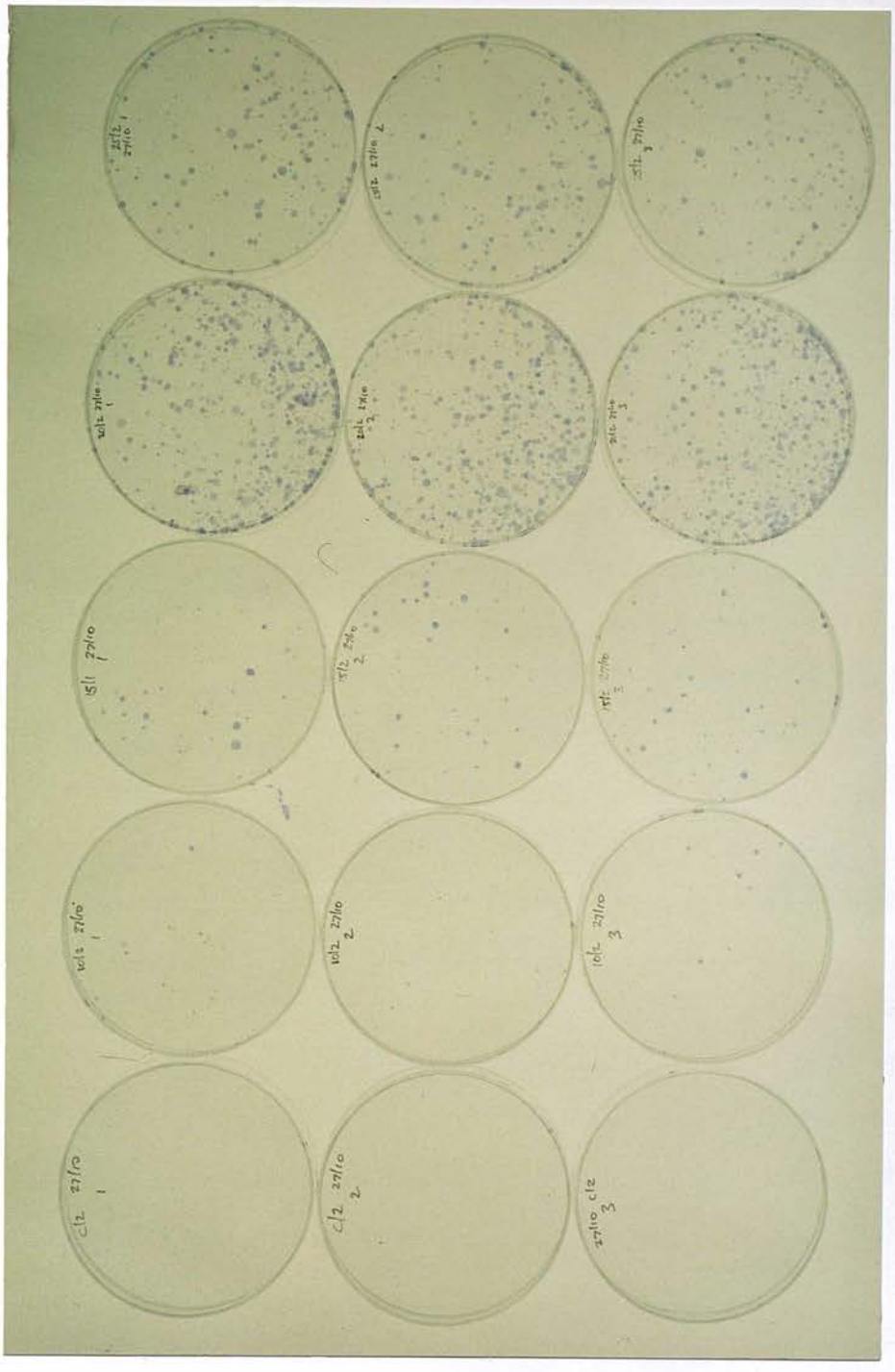


Fig. 4.27: Optimisation of calcium phosphate-mediated transfection (Chen and Okayama, 1987): Mass of DNA: Colonies stained with Giemsa following transfection with pPURO and selection in 2μg/ml puromycin. (Bottom to Top Rows) Control transfection with non-relevant selectable marker (pBKCMV); Transfections with pPURO: 10μg; 15μg; 20μg; 25μg.

Table 4.4. Optimal Plasmid DNA Concentrations.

Plasmid	Optimal Mass of DNA (μg)
pPURO	20
pBabeNeoVP16GalER^{tm}	30
p3'SS	20
pOPRNedd2	30
pSP65GCNedd2	20
pSP65GCNedd2puroSD	20
pSP65GCp53wtpuro	30
pSP65GCWAF1-143puro	30
pCMV-βgal + pPURO	20 (5 + 15)

4.2.2.1.4 Estimation of Transfection Efficiency.

0.5×10^6 cells were plated 24 hours prior to transfection with 20μg of pPURO with 2 x BBS at pH 6.95. The precipitate was washed off and the cells were incubated in normal medium for 24 hours. The cells were then split 1 in 10 and replated in selective medium. In parallel, 1×10^3 transfected cells were plated in replicate plates in normal medium in order to determine the plating efficiency. Colonies were fixed and stained after 10 days in culture. The transfection efficiency was calculated according to the following formula:

$$\text{Transfection Efficiency} = \frac{\text{Mean Number of Colonies x Dilution Factor}}{\text{Number of cells Transfected x Plating Efficiency}}$$

$$= \frac{513 \quad x \quad 10}{0.5 \times 10^6 \quad x \quad 0.683}$$

$$= 0.0150 = 1.5\%$$

The transfection efficiency under optimal conditions with pPURO in Rat1 using the Chen and Okayama version of the calcium phosphate coprecipitation method was therefore estimated to be 1.5 %. This is in close agreement with transient transfection experiments with pCMV-βgal in which cells were fixed and stained with X-gal 24 hours following transfection. 0.9% of cells in such experiments stained positive for β–galactosidase.

4.3 Results

4.3.1 Evaluation of the *lac* repressor / operator vector system.

4.3.1.1 Generation of Rat1 cells expressing LacI-NLS.

As an initial step towards lacI-regulable expression of genes such as p53, $p21^{WAF1/CIP1}$, c-*myc* and *nedd2*, the Rat1A fibroblast cell line was engineered to express the modified LacI-NLS repressor protein. It was expected that the expressed protein would be identifiable by immunofluorescence microscopy and localised to the nucleus of transfected cells. Plasmid p3'SS, the expression vector that contains the *lac*I-NLS construct, was transfected into Rat1 cells and stable transfectants were selected for on the basis of hygromycin resistance. Individual clones were picked, expanded and analysed for expression of the LacI protein by immunofluorescence microscopy. Each of the clones was scored for total LacI expression and for subcellular distribution (Table 4.5). Fig. 4.28 shows one clone (Rat1Rep54) with strong nuclear staining using the anti-LacI antibody. Other sub-lines also had nuclear staining but Rat1Rep54 was chosen as the recipient line for as it qualitatively showed the best nuclear localisation and strongest antibody staining of the 19 lines tested.

4.3.1.2 Transfection of IPTG-inducible vectors into Rat1Rep54 cells.

4.3.1.2.1 Attempted generation of lac-regulable p21WAF1-143 expressing Rat1 cells.

In order to test whether the Lacswitch system was capable of controlling the expression of a gene known to inhibit cell growth such that a reversible growth arrest could be induced in Rat1 cells, attempts were made to generate stable cell lines that expressed the truncated $p21^{WAF1/CIP1}$ insert containing only the cyclin-CDK-inhibitory domain under the *lac*I-NLS fusion protein. Plasmid pOPRWAF1-143 was transfected into Rat1Rep54 cells and selected for on the basis of G418 resistance in

the absence of IPTG. However, clones were generally very small (less than 50 cells per clone) and often consisted of large, poorly packed, flat cells. Very few mitotic cells were identified. Attempts were made to expand individual clones but these did not result in the generation of any stable cell lines.

4.3.1.2.2 Attempted generation of lac-regulable myc-ER expressing Rat1 cells.

In order to generate Rat1 cells engineered to overexpress c-*myc* under the transcriptional control of an inducible promoter, attempts were made to generate cell lines expressing the *myc*ER fusion gene dependent upon the presence of IPTG in the medium. Such a cell line would be expected to exert tight regulation of c-*myc* activity owing to control at both the transcriptional (IPTG) and post-transcriptional (β-oestradiol) levels. Accordingly, pCMVOPMER (containing the mycER fusion gene under the control of a *lac*I-regulable CMV promoter) was transfected into Rat1Rep54 cells and selected for upon the basis of puromycin resistance. The transfection efficiency of such transfection experiments was unexpectedly very low. Only a few (5-8) colonies formed on each plate and although these colonies appeared healthy in normal culture medium and increased in size during selection, attempts to expand these in medium containing charcoal-stripped neonatal calf serum purchased from Sigma resulted in no stable cell lines. These results may be interpreted as indicating that the *lac* control of myc-ER expression was incomplete and possibly lead to a reduction of cell viability under the stressful conditions of selection and cloning in tissue culture. However, the above experiments should be regarded as inconclusive as it was subsequently found that the plating efficiency of Rat1 cells in the batch of serum used was observed to be very low at low plating densities: similar conditions to which cells subjected to selective antibiotics are exposed (data not shown).

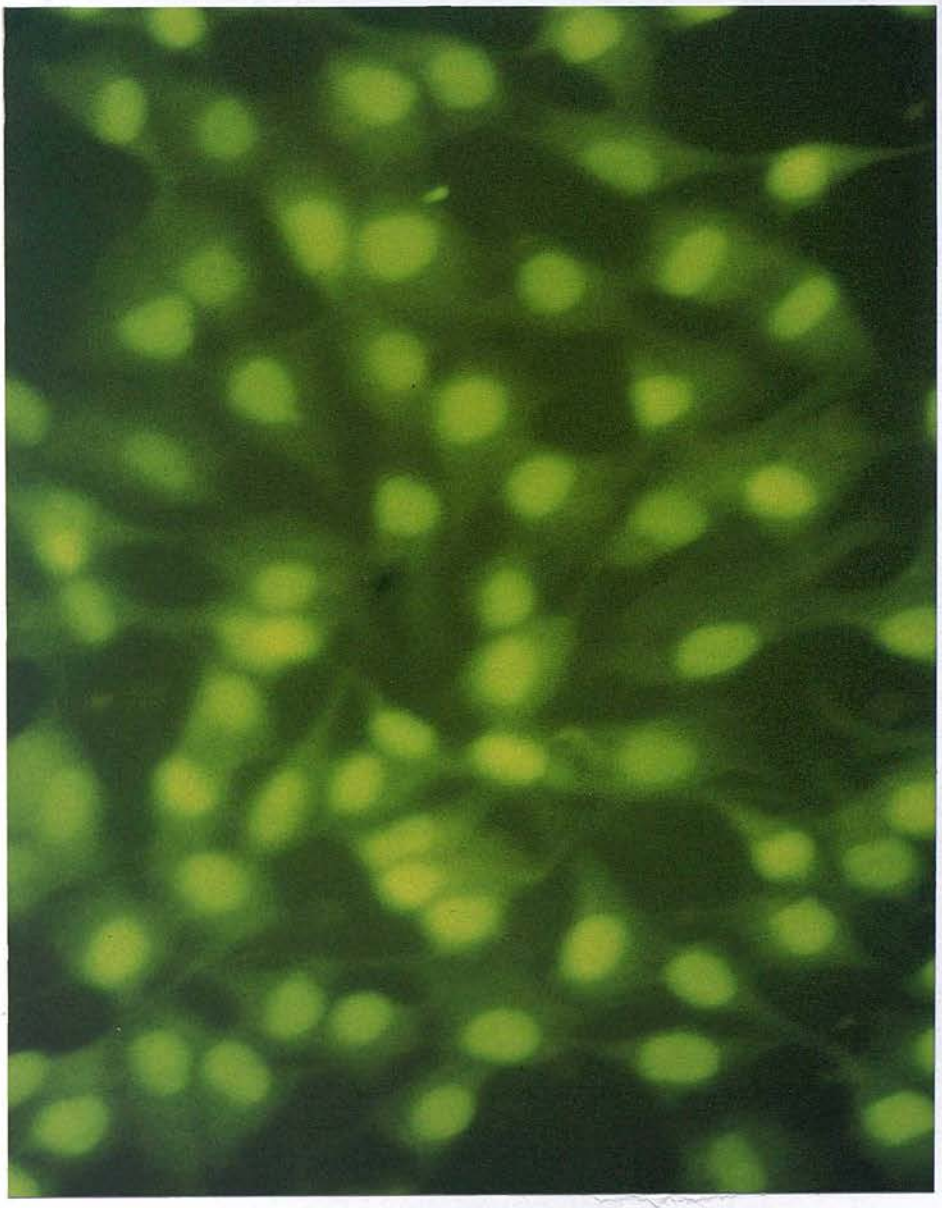


Fig. 4.28: LacI Immunofluorescence of Rat1 cells stably-transfected with p3'SS (clone: Rat1Rep54). Note that the majority of LacI-NLS expression is localised to the nucleus.

Table 4.5. LacI Immunofluorescence.

Clone	Total LacI staining	Nuclear localisation
Rat1Rep3	weak	+
Rat1Rep6	weak	++
Rat1Rep7	weak	+++
Rat1Rep30	weak	++
Rat1Rep54	v. strong	+++
Rat1Rep56	strong	+++
Rat1Rep58	strong	++
Rat1Rep3.1	strong	++
Rat1Rep3.2	strong	++
Rat1Rep3.4	strong	+++
Rat1Rep3.5	strong	+
Rat1Rep3.7	weak	-
Rat1Rep3.8	weak	++
Rat1Rep3.9	weak	+
Rat1Rep3.10	weak	+
Rat1Rep3.12	strong	+
Rat1Rep3.13	weak	-
Rat1Rep3.15	weak	++
Rat1Rep3.18	strong	+++

4.3.1.2.3 Attempted generation of lac-regulable p53 expressing Rat1 cells.

Pilot transfection experiments showed that plasmid pOPRp53wt was capable of forming colonies following transfection of Rat1Rep54 cells and selection with G418. Colonies appeared to be healthy by phase contrast microscopy and were able to be expanded. It was expected that overexpression of p53 would cause alteration of cell cycle activity in rat fibroblasts. In order to test the ability of LacI protein to suppress expression of p53 from vectors containing *lac* operator sites, either of two vectors, pOPRp53wt or pBKOP53wt, which contain 2 or 3 operator sites respectively, were transfected into either Rat1Rep54 or the parental Rat1 cell line. If p53 expression was suppressed by LacI expression, it was expected that more colonies would form following transfections of Rat1Rep54 cells than in transfections of RatI cells. Accordingly, colonies on replicate plates were stained with Giemsa and counted 14 days following transfection. Table 4.6 shows the results of these experiments. There was no statistically-significant difference between the number of transfectant colonies derived from lacI-expressing Rat1Rep54 and parental Rat1 cells in experiments with either of the two inducible p53 expression vectors.

It was noted that pBKOP53wt consistently produced fewer colonies following selection than pOPRp53wt. There was no significant difference between the number of colonies obtained by transfection of Rat1Rep54 cells and Rat1 cells with either pOPRp53wt or pBKOP53wt. Although p53 mRNA or protein levels were not measured in this work, it is possible that repression of expression of wild-type p53 does not affect the efficiency of colony formation of Rat1 cells.

Table 4.6. Number of colonies (±SEM) derived from transfections with IPTG-inducible p53 expression vectors.

Cell line	pOPRp53wt	pBKOP53wt
Rat1	45.5 ± 8.7 n = 6	17.2 ± 5.1 n = 6
Rat1Rep54	51.8 ± 5.4 n = 6	12.0 ± 3.75 n = 6

4.3.1.2.4 Repression of expression of Nedd2 by lacI-NLS is not complete.

In order to test whether repression by *lac*I-NLS of an ICE-like protease gene (Nedd2) under the control of a promoter containing *lac* operator sites was sufficient to allow construction of stable cell lines, plasmid pOPRNedd2 or a control vector (pOPRMT) lacking the Nedd2 expression cassette was transfected into Rat1Rep54 cells. Cells transfected with the control plasmid formed large, easily expandable colonies upon selection with G418 at a transfection efficiency of 0.41%. However, cells transfected with pOPRNedd2 formed only a few small, colonies (less than 100 cells per colony) which contained many gaps with apoptotic cells and which did not survive extensive attempts at expansion. In a transient transfection assay, Nedd2 mRNA expression was detected by RT-PCR after 9 hours in DNase-treated, total RNA samples from cells transfected with pOPRNedd2 in both the presence or absence of IPTG (Fig. 4.29a). It was concluded that cells transfected with pOPRNedd2 were killed by the expression of Nedd2 caused by incomplete repression of transcription by LacI.

4.3.2 Evaluation of the Tamoxifen-Sensitive GalER Expression System

4.3.2.1 Generation of Rat1 Sublines Expressing the VP16GalER^{tm} Transactivator.

The first stage of the construction of cell lines designed to express exogenous genes conditional upon the addition of 4-hydroxytamoxifen to the medium was to create a subclone of the Rat1 cell line that expressed the VP16GalER^{tm} transactivator. Accordingly, pBabeNeoVP16GalER^{tm} was transfected into Rat1 cells and selected by G418 resistance. Six sublines were tested for expression of the VP16GalER^{tm} fusion transcript by Northern blotting. (Fig. 4.29b). Note that although 10μg of total cellular RNA was loaded in each lane, only a weak signal from the 4.4kb RNA transcript was detectable in 4 out of the 6 clones. Clone Rat1/4.7, which expressed the VP16GalER^{tm} RNA at intermediate levels was chosen for further study with Gal4-inducible vectors. Clone Rat1/4.12, which showed the highest levels of expression, grew relatively slowly in culture and was not used further.

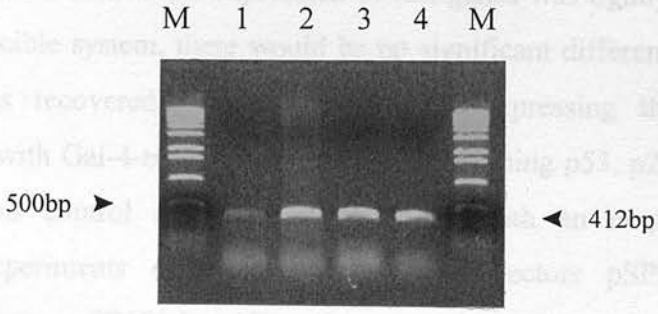


Fig. 4.29a: *Nedd2* RT-PCR of Rat1Rep54 and Rat1 cells transiently transfected with pOPRNedd2. Note that *nedd2* mRNA is detected in all lanes. Control (untransfected cells or no cDNA template) PCR reactions were blank (on a separate portion of the gel). .M: Life Tech. 1kb ladder. Lane 1: Rat1Rep6.4 cells transfected with pOPRNedd2, -IPTG; Lane 2: Rat1Rep6.4 cells transfected, +IPTG; Lane 3: Rat1 cells transfected , -IPTG. Lane 4: Rat1 cells transfected , +IPTG.

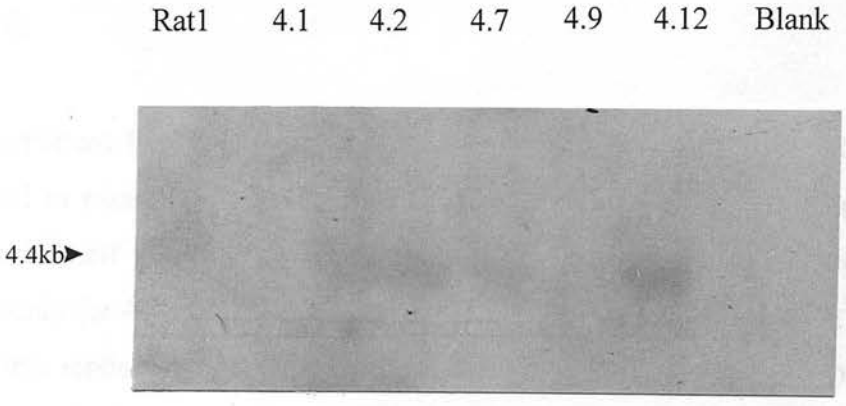


Fig. 4.29b: Northern analysis of Rat1 cells stably-transfected with pBabeNeoVP16GalER^{tm}. An HindIII probe internal to the VP16GalER^{tm} insert was generated from pBabeNeoVP16GalER^{tm} and was used to probe a total RNA Northern blot of Rat1 cells and transfectants. Although the signal was generally weak, several clones were positive and clones 4.12 and 4.7 showed the greatest expression of VP16GalER^{tm} RNA.

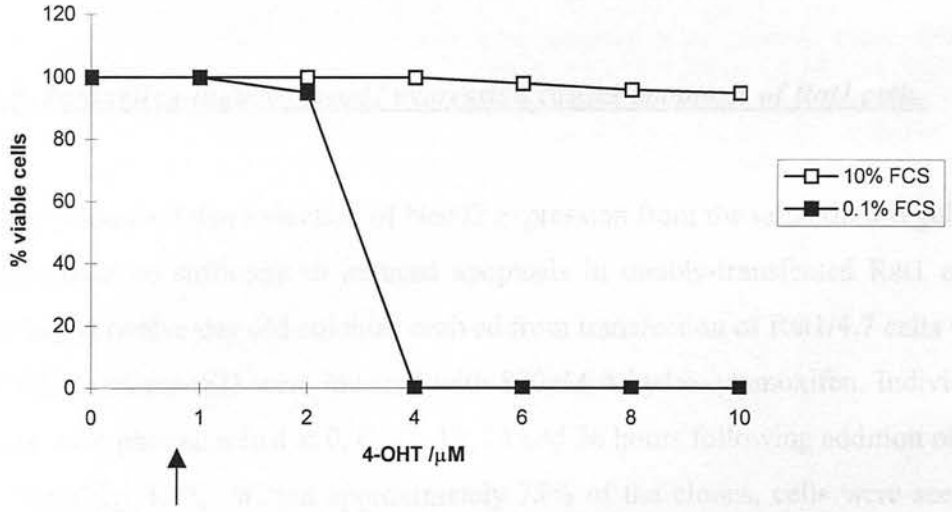
4.3.2.2 Testing of Gal4-inducible constructs in Rat1 cells.

It was hypothesised that, if the expression of test genes was tightly regulated in the tamoxifen-inducible system, there would be no significant difference in the number of transfectants recovered from Rat1/4.7 cells (expressing the VP16GalER^{tm} transactivator) with Gal-4-responsive constructs containing p53, p21 or c-*myc* genes as compared to control experiments performed with an empty vector. Pilot transfection experiments of Rat1/4.7 cells with vectors pSP65GCp53wtpuro, pSP65GCmycpuro, pSP65GCWAF1-143puro and pSP65GCWAF1-160puro all produced similar numbers of healthy, expandable clones to that of transfections with pSP65GCpuro, a control vector not containing an inserted gene (approximately 40-50 colonies per plate). Similarly, transfections of pSP65GCp53wthygro produced similar numbers of colonies to pSP65GChygro. These data indicate that there is no selection against colony formation by Gal4-inducible vectors containing apoptosis or growth inhibitory genes in the absence of active VP16GalER^{tm} transactivation.

4.3.2.3 Dose-Response Relationship of 4-hydroxytamoxifen upon Rat1 Cell Toxicity.

It is important for the validity of experiments with inducible expression systems designed to express apoptosis promoting genes to be sure that the inducing agent does not itself possess toxic properties. Accordingly, a dose-response toxicity relationship for 4-hydroxytamoxifen in Rat1 cells was carried out. 1 x 10^5 cells were plated into replicate wells of a 6 well tissue culture plate and allowed to attach to the substratum for 24 hours. The medium was then replaced with medium containing various concentrations of 4-hydroxytamoxifen (4-OHT) (Sigma). The proportion of live cells after a further 24 hours in culture was determined by counting of cells in several fields from each well by phase contrast microscopy. Because serum deprivation experiments with vectors expressing c-*myc* were planned, the experiment was performed in parallel with medium containing 0.1% serum. (Fig. 4.30). At the concentration of 4-OHT used for induction experiments (800nM), there appeared to

Fig. 4.30. Toxicity of 4-hydroxytamoxifen. (Arrow: concentration (800nM) used in induction experiments)



4.3.2.4 Generation of Rat1 sub-lines with tamoxifen-inducible Nedd2

Rat1/4.7 cells expressing the VP16GalER^{tm} fusion gene were transfected with pSP65GCNedd2SDpuro and selected with puromycin for up to 2 weeks. These clones were large and could be expanded as single colonies. RNA and protein were extracted at 6 hour intervals from pools of clones induced with 800nM 4-hydroxytamoxifen (Sigma). Induction of Nedd2 expression was monitored by RT-PCR of DNAse-treated RNA samples (Fig. 4.31a). Nedd2 mRNA was detected within 6 hours. At later time points RNA samples showed evidence of degradation (not shown) although mRNA was still detectable by RT-PCR at 36 hours.

4.3.2.5 Tamoxifen-induced Nedd2 expression causes apoptosis of Rat1 cells.

It was hypothesised that induction of Nedd2 expression from the tamoxifen-regulable vectors would be sufficient to induced apoptosis in doubly-transfected Rat1 cells. Accordingly, twelve day old colonies derived from transfection of Rat1/4.7 cells with pSP65GCNedd2puroSD were induced with 800nM 4-hydroxytamoxifen. Individual colonies were photographed at 0, 6, 12, 18, 24 and 36 hours following addition of the tamoxifen (Fig. 4.32). Within approximately 75% of the clones, cells were seen to round up and detach from the substratum between 6 and 12 hours. At the 18 hour time point the majority of cells in these clones had detached and formed spherical, highly refractile apoptotic bodies. By 24 hours all cells in these clones were dead. Apoptosis was confirmed as the mode of cell death by acridine orange staining (not shown) or electron microscopy of dead cells collected from the medium (Fig 4.31b). Clones that showed no initial response to tamoxifen were unaffected and continued to increase in size throughout the experiment. At the dose of tamoxifen used, untransfected Rat1, Rat1/4.7 cells or Rat1/4.7 cells transfected with empty vector pSP65GCpuro were unaffected by addition to the medium of 800nM 4-hydroxytamoxifen or an equivalent volume of the ethanol vehicle (not shown).

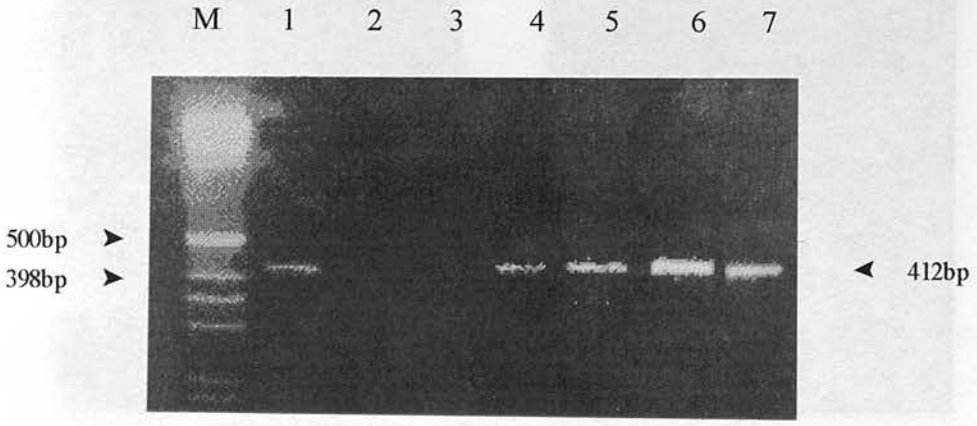


Fig. 4.31a: RT-PCR analysis of 4-hydroxytamoxifen-induced *nedd2* expression in Rat1/VP16GalER^{tm} cells (Rat1 4.7) transfected with pSP65GCNedd2puroSD. M: Life Tech. 1 kb ladder. Lane 1: PCR positive control (reaction spiked with 1pg pSP65GCNedd2puroSD plasmid DNA). Lane 2: PCR blank (no cDNA template); Lane 3: cDNA from uninduced cells (0 hours). Lane 4: 6 hours post induction. Lane 5: 12 hours; Lane 6: 18 hours; Lane 7: 24 hours.

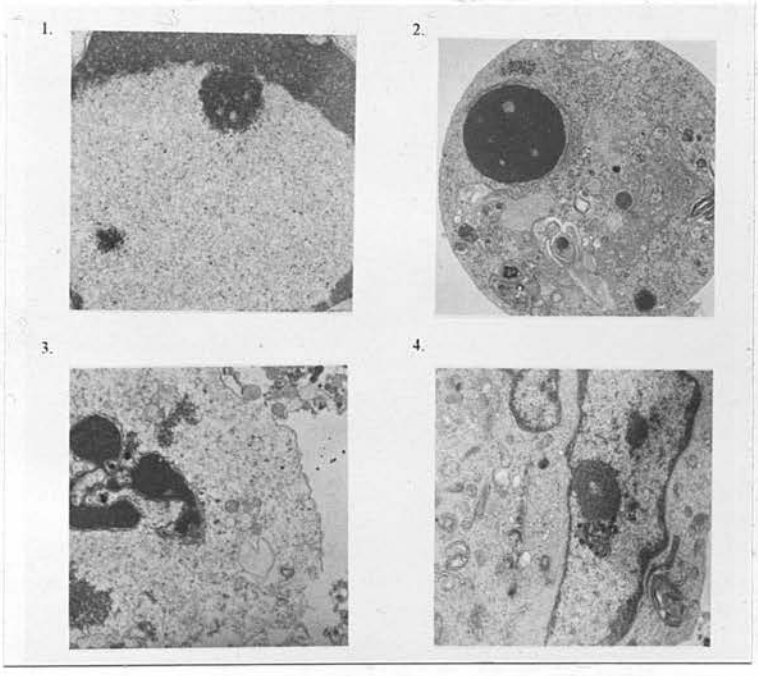


Fig. 4.31b: Transmission electron microscopy of Rat1 4.7 / pSP65GCNedd2puroSD transfectants induced with 4-hydroxytamoxifen. (osmium tetroxide post-fix; uranyl acetate / lead citrate stain) 1: Early stage apoptotic nucleus showing marginated, condensed chromatin and nucleoli. (x800) 2: Apoptotic body with condensed nuclear fragment. Note the whorls of membrane and dilated endoplasmic reticulum in the cytoplasm. (x600). (Contrast in size with normal cell at same magnification in 4.) 3: Late stage apoptotic cell with fragmented, condensed nucleus and secondary necrosis with rupture of the cell membranes. (x620). 4: Normal Rat1 cell. (x 600).

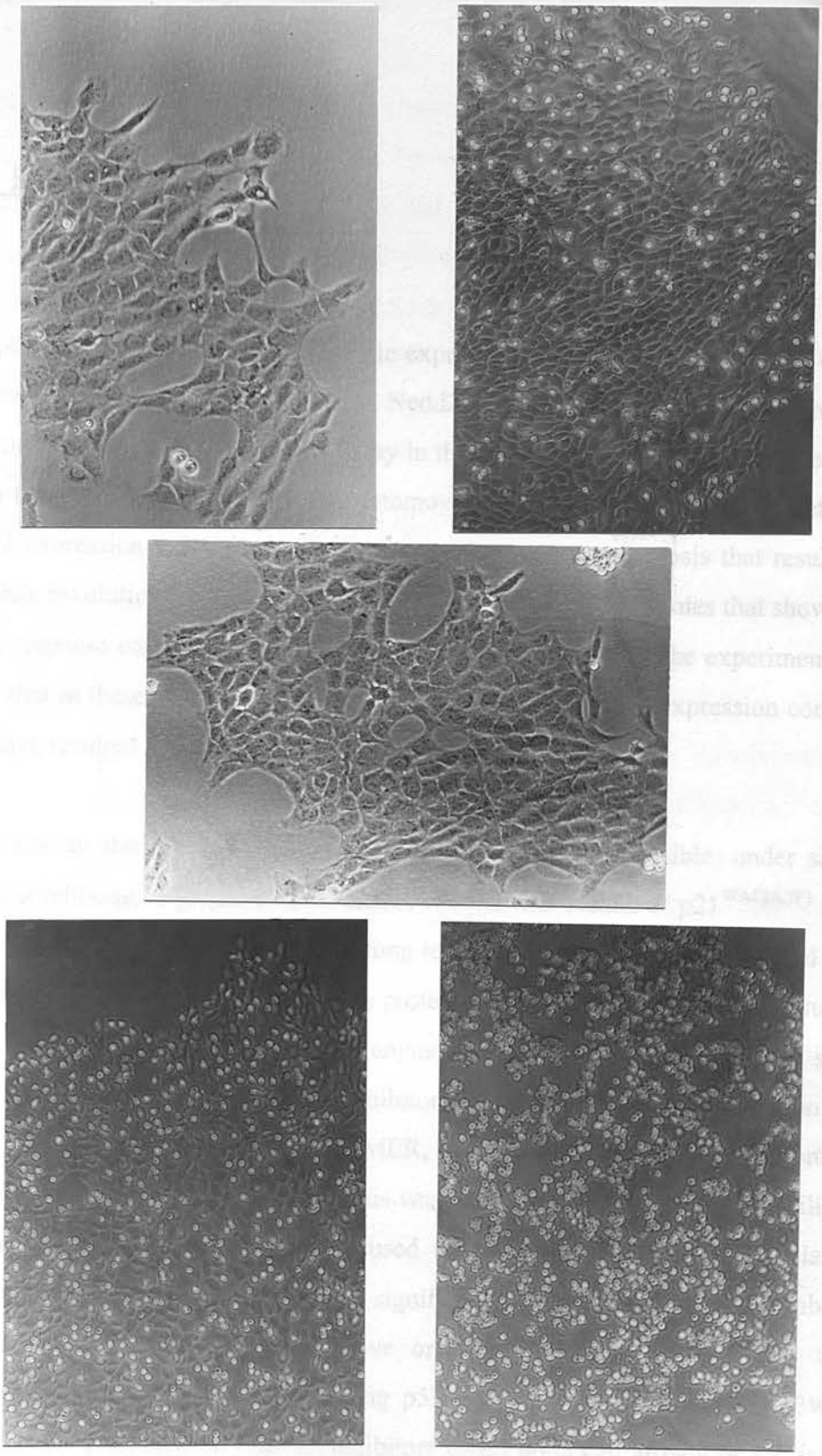


Fig. 4.32: Phase contrast microscopy of Rat1 4.7 / pSP65GCNedd2puroSD transfectant colonies induced with 800nM 4-hydroxytamoxifen. Top left: Untreated (x100); Top right: 6 hours (x40); Bottom left: 12 hours (x40). At 24 hours all cells had rounded-up and detached (Bottom right). Centre: Rat1 4.7 cells transfected with empty vector (pSP65GCpuro) treated with 800nM 4-hydroxtamoxifen, 24 hours (x100).

4.4 Discussion.

A modification of the Gal-ER inducible expression system was used here to control expression of the ICE-related protease Nedd2 in Rat1 fibroblasts. Nedd2 transcripts were not detectable by an RT-PCR assay in the uninduced state but were detected six hours following addition of 4-hydroxytamoxifen to the medium. This induction of Nedd2 expression coincided with the onset of a wave of apoptosis that resulted in complete involution of transfected colonies by 24 hours. The colonies that showed no initial response continued to be unaffected during the course of the experiment. It is likely that in these surviving colonies, integration of the Nedd2 expression construct may have resulted in disruption of the Nedd2 coding sequence.

In contrast to the tamoxifen-inducible system, it was not possiible, under similar culture conditions, to produce stable clones of cells with Nedd2 or $p21^{WAF1/CIP1}$ under the control of the Rous sarcoma virus long terminal repeat in vectors designed to be repressible by a modified *lac* repressor protein. This suggests that such a system is inherently too leaky to be of use in demanding situations such as with the stable expression of cell death or cell cycle inhibitory genes. Surprisingly, transfection with myc-ER expression vector, pCMVOPMER, also did not produce stable clones in 'oestrogen-free' conditions, although this was eventually ascribed to the inability of the batch of charcoal-stripped serum used to support growth of Rat-1 cells. An unexpected result was that there was no significant difference between the number of colonies produced in repressor positive or repressor negative Rat1 cells upon transfection with *lac* operator-containing p53 expression plasmids pOPRp53wt or pBKOP53wt. Wild-type p53 has an inhibitory effect upon cell growth or survival in most cells and it seemed plausible that enforced p53 expression in the absence of repressor from these vectors would substantially decrease the transfection efficiency relative to repressor positive cells. There are several possible explanations for this

observation. Firstly, the vectors may contain mutant p53 as a result of a cloning artifact. This possibility was rejected by sequencing the p53 inserts in the two vectors. Secondly, even if p53-transfected Rat-1 cells do overexpress p53, p53 expression may have no effect upon cell growth or apoptosis. This may be because of the species difference between the exogenous murine p53 and the rat cells used. However, murine p53 has previously proven to be effective in rat embryo fibroblasts (Michalovitz *et al,* 1990). Alternatively, as it known that Rat-1 cells possess endogenous wild-type p53, it is possible that these cells have mutations in other genes, downstream or at the activation of p53, that may cause these cells to be unresponsive to the addition of exogenous p53. However, the one previous report in which Rat-1 cells have been transfected with constitutive murine p53 expression constructs (in co-transfections with separate plasmids containing a selectable *neo* gene), exogenous wild-type p53 reduced the numbers of clones surviving selection relative to parallel transfections with point or deletion mutants. Exogenous wild-type p53 protein was not detected in the surviving clones, most of which did not contain an integrated copy of the p53 expression vector or had less than one copy per cell (Finlay *et al,* 1989). If indeed p53 mRNA was expressed from the vectors in Rat-1 cells, a third possibility is that p53 translation was inhibited. The p53 cDNA used contains the full 5' untranslated region of the endogenous mRNA which has been suggested to form an extensive stem loop secondary structure, thereby inhibiting translation (Mosner *et al,* 1995). The exogenous p53 protein may therefore be under the same translational control as endogenous protein and therefore expressed at low levels in Rat-1 cells. Activation of the exogenous p53 may therefore require a stimulus such as DNA damage or oncogene activation in order to alter protein stability and therefore p53 protein concentration. This could be tested by comparing both the mRNA and protein levels in the transfectants with that in untransfected Rat-1 cells.

A similar repression system to the *lac* operon, based upon the *tet*-repressor, *tet*R, has not been used in mammalian cells as it is believed that steric inhibition of RNA polymerase-mediated strand elongation alone is not sufficient to control transcription

effectively in the case of low *tet*O occupancy. Higher occupancy rates are not achievable in mammalian cells as *tet*R is toxic at greater concentrations than are generally achievable (Gossen and Bujard, 1993). The *lac*-repressor protein used here can be expressed at easily detectable levels and is localised mainly in the nucleus as a result of fusion with an SV40 nuclear localisation signal peptide. Therefore, operator occupancy by LacI-NLS is unlikely to be the limiting factor in this case. The data presented here suggests that inhibition of transcription by the *lac* repressor at operator sites placed within and (or) downstream of a strong viral promoter is incomplete. Indeed, following transient transfection of pOPRNedd2 into cells expressing LacI-NLS, Nedd2 mRNA was detected by RT-PCR even in the absence of the inducer, IPTG.

The production of clonal cell lines with tamoxifen-inducible Nedd2 expression will allow biochemical dissection of the mechanisms by which Nedd2 causes apoptosis. In particular, these cell lines may provide a tool to identify substrates that are specifically cleaved by the Nedd2 cysteine protease activity. Although not shown in this work, it is likely that cells stably-transfected with inducible constructs for ICE-related proteases are likely to achieve induced protease molecule concentrations at least 1000-fold lower than that in experiments where loading of cells with proteases such as trypsin or proteinase K resulted in cell death resembling apoptosis as a result of non-specific cleavage of many cellular proteins (Williams and Henkart, 1994). This much lower final concentration of protease in transfected cells is much more likely to result in apoptosis as a result of cleavage of substrates at specific sites. Further work will be needed to determine whether, like tTA-inducible expression, tamoxifen-inducible Nedd2 protein levels can be titrated by varying the inducer concentration. If this is indeed the case, then the VP16GalER^{tm} cell lines developed here may provide a means to circumvent artifacts resulting from non-specific cleavage of substrates by the gratuitous overexpression of ICE-like proteases.

One potential side-effect of the use of the strong VP16 *trans*-activation domain in fusion proteins is squelching of transcription factors acting at endogenous genes.

Both Gal4-VP16 and VP16-ER proteins, when transiently transfected, have been shown to inhibit transcription from promoters not containing Gal-4 binding sites (Gilbert *et al,* 1993; Sadowski *et al,* 1988). The VP16 domain is common to both the VP16GalER and tTA transactivators as well as chimaeric *lac* repressors containing the VP16 moiety (Baim *et al,* 1991). Therefore work needs to be done to determine whether these proteins cause squelching in stable transfectants. Squelching could account for the barely detectable levels of VP16GalER^{tm} mRNA in transfected Rat1 cells. Use of tetracycline *trans*-activator vectors in mammalian cells invariably results undetectable tTA expression and requires laborious characterisation of individual candidate tTA lines by transient transfection with a tetracycline inducible reporter gene. Luckily, this was not required here as transient transfection efficiency of Rat1 cells was no higher than 1% when determined by histochemical staining for β-galactosidase. Repeated attempts to measure the activity of CAT in such transient assays failed to achieve levels much above background.

Notwithstanding the comments above, the tamoxifen-inducible system described here provides sufficiently tight regulation of gene expression to allow generation of stable cell lines with apoptosis-promoting genes. Charcoal-stripping of steroid hormones from serum was not a requirement to maintain transfected cells in the uninduced state. The use of the tamoxifen-sensitive mutant oestrogen receptor is therefore a major refinement of the VP16Gal-ER *trans*-activator that will enhance its utility in transgenic animals where the normal high concentration of endogenous oestrogenic steroids would otherwise result in significant background expression of the transgene. This is particularly important if the result of expression of the transgene is likely to result in ablation of the tissues where the transgene is expressed. The use of the tamoxifen-inducible system should therefore facilitate the study of apoptosis.

Chapter 5.

5. Concluding Discussion.

Evidence was presented here to suggest that, at least in one particular cellular context, expression of wild-type p53 can protect cells from the lethal effects of the DNA damaging agents etoposide and bleomycin. This was probably mediated by enforcement of a G0/G1 cell cycle arrest, thus allowing time for recovery from the insult. As certain cell types, including fibroblasts, undergo a prolonged growth arrest following DNA injury, the p53-dependent growth arrest is likely to provide enough time for DNA repair to proceed to completion. In contrast, in the presence of mutant p53 expression, the fibroblast cell lines studied here underwent apoptosis associated with an accumulation of cells in G2/M. The p53-independent apoptosis in this situation may be the cellular response to abortive attempts to undergo mitosis with unrepaired DNA.

The response to induction of wild-type p53 activity in other cellular situations does not always result in cell survival and growth arrest. Often cells become committed to undergoing apoptosis, with or without passing through a state of growth arrest. The irradiated thymocyte is a good example of this. In the absence of p53, thymocytes often give rise to thymic lymphomas *in vivo* and are unresponsive to irradiation *in vitro* (Clarke *et al,* 1993; Lowe *et al,* 1993). Moreover, this death pathway can be separated from the growth arrest pathway. This is exemplified by the defect in DNA damage-induced fibroblast growth arrest but not thymocyte apoptosis in mice deficient for the p53-inducible p21 gene (Brugarolas *et al,* 1995; Deng *et al,* 1995). Therefore the response to p53 is determined by cell lineage.

Other factors may affect the outcome of activation of wild-type p53 including availability of certain growth factors or the expression of other genes, especially oncogenes. It is known that activated *ras* can alter susceptibility to apoptotic stimuli (Arends *et al,* 1993). For this reason, *myc* and p53 co-transfection experiments were

attempted in both the ras-transformed Clone 6 cell line and the spontaneously immortalised, but non-transformed cell line, Rat1. However, there was no obvious difference in results between the two cell lines in these experiments. In this report, data was presented that expression of wild-type p53 in fibroblasts could result in reduced viability if co-transfected with plasmids expressing c-*myc*, suggestive of a co-operative relationship in the control of cell death. This is supported by observations by others that apoptosis induced by mitogenic genes, including c-*myc*, E2F-1 and adenovirus E1A cannot occur in cells deficient for p53 (Kowalik *et al,* 1995; Lowe *et al,* 1994; Hermeking and Eick, 1994; Wagner *et al,* 1994). Therefore, p53-dependent apoptosis is likely to be a more important determinant of tumour suppression than p53-dependent growth arrest, acting to delete cells of probably all lineages that carry activated oncogenes. This is evidenced by the unexpectedly very low rate of neoplasia in p21 -/-mice (Brugarolas *et al,* 1995; Deng *et al,* 1995) and by the observation that certain mutant p53 alleles that are unable to induce apoptosis when overexpressed in an appropriate test line also have lost the ability to suppress cellular transformation, despite retaining the ability to cause transcriptional activation of p21 and growth arrest (Friedlander *et al,* 1996; Ludwig *et al,* 1996; Rowan *et al,* 1996).

Inducible expression systems should allow the detailed analysis of deleterious genes in stable cell culture lines. In this project a Nedd2 cDNA was cloned as a test gene downstream of two different inducible promoters: one containing *lac* operator sites in which expression is downregulated by binding of a repressor protein until the addition of IPTG; and a second promoter that becomes active only in the presence of 4-hydroxytamoxifen. Whilst the *lac*-repressor-based system was leaky, resulting in only transient expression, the VP16GalER^{tm} provided sufficiently tight control of expression to enable the propagation of stably-transfected cells which underwent apoptosis upon induction with 4-hydroxytamoxifen. Little is known of the substrate preferences of this relatively poorly characterised protease. The tamoxifen-inducible system and the Nedd2 cell lines generated here are therefore a powerful tool for the study of apoptosis effector mechanisms.

Scientific method involves making perturbations in identified factors in order to learn about the normal from those alterations. In molecular cell biology this can often require manipulations that cause the overexpression of a particular gene product or mutation that eliminates or alters the function of a gene. A common strategy for specific genetic manipulation is gene knockout by vector-directed homologous recombination in totipotent embryonic stem cells. This is the first step in the derivation of recombinant animals that have a targeted deletion at a particular genomic locus. This strategy has been useful for determining the effects of absence of particular genes. However ideal this strategy appears to be as a tool of science, the function of a knocked-out gene can only be *inferred* by careful analysis of the phenotype of the targeted animals and their cells in comparison with normal animals. Indeed, many gene knockouts result in remarkably similar phenotypes or are embryonic lethal. Further, deletion of a gene whose function is redundant *in vivo* can result in a normal phenotype. Therefore, in the real scientific world, information about a particular gene, by necessity, is drawn from several sources including expression experiments. Despite arguments that overexpression is an artificial situation, the ability to exogenously supplement the expression of a gene is still of equal scientific value and as much ingenuity as has been poured into gene targeting techniques is required to develop expression technology. Expression techniques are, after all, the basis of future gene specific therapies.

Appendix A: Solutions

TE
10 mM Tris (pH8.0)
1 mM EDTA

PBS
8g NaCl
0.2g KCl
1.44g Na_2HPO_4
0.24g KH_2PO_4
in 1 litre DDW pH7.4 autoclaved

(x10) TAE
48.4g Tris
11.42 ml stock glacial acetic acid
3.72g EDTA
make up to 1 litre with DDW (pH 8).

(x10) TBE
108g Tris
55g boric acid
40 ml 0.5M EDTA
make up to 1 litre with DDW (pH 8).

dNTP mix.
10µl of each 100mM stock dNTP.
To 100µl with DDW.
Store at -20°C.

(x10) gel loading buffer
0.25% bromophenol blue
25% ficoll
in DDW

SOB medium
20g Bacto-tryptone
5g Bactro-yeast extract
0.5g NaCl
in 1 litre DDW autoclaved

SOC medium
1 litre SOB
5 ml 2M $MgCl_2$
20 ml 1M glucose
filter sterilise (0.2µm filter)

LB medium
10g Bacto-tryptone
5g Bacto-yeast extract
10g NaCl
in 1 litre DDW
pH7.5 autoclaved

LB agar
7.5g bacto agar
500 ml LB medium
autoclave
cool to 50°C
add antibiotic (e.g. 100µg/ml ampicillin)

2xYT Broth
10g NaCl
!0g Yeast Extract
16g tryptone
to 1 litre with DDW (pH 7.5 with NaOH), autoclave.

Miniprep Cell resuspension solution
50 mM Tris-HCl pH7.5
10 mM EDTA
100µg/ml RNaseA

Miniprep Cell lysis solution
0.2M NaOH
1% SDS

Miniprep Neutralization solution
1.32M Potassium acetate pH4.8

Miniprep Column wash solution
200 mM NaCl
20 mM Tris-HCl pH7.5
5 mM EDTA

dilute with 95% ethanol to a final ethanol concentration is 55%

Maxiprep resuspension buffer: P1
100µg/ml RNaseA
50 MM tris/HCl
10 mM EDTA pH8.0

P2: lysis buffer
200 MM NaOH
1% SDS

P3: neutralisation buffer
3M KAc pH5.5

QBT: equilibration buffer
750 mM NaCl
50 mM MOPS
15% ethanol pH 7
0.15% triton X-100

QC: wash buffer
M NaCl
50 mM MOPS
15% ethanol pH8.5

QF: elution buffer
1.25M NaCl
50 mM tris/HCl
15% ethanol pH8.5

2x BES-buffered solution (BBS)
50 mM *N,N-bis*(2-hydroxyethyl)-2 aminoethanesulfonic acid (BES)
280 mM NaCl
1.5 mM Na_2HPO_4 pH 6.95
800 ml DDW
adjust to appropriate pH (6.95 - 6.98) with NaOH
filter sterilise and freeze in 20 ml aliquots at -20°C

2.5M $CaCl_2$
183.7g $CaCl_2$ dihydride
DDW to 500 ml
filter sterilise and store in 10 ml aliquots at -20°C

10x MOPS running buffer
1.8g MOPS (3-N Morpholinopropanesulphonic acid)
3.72g EDTA (Disodium salt)
4.1g Sodium acetate
to 1 litre with DEPC treated water
pH 7.0
0.2µm filter

10x Ligase buffer
300mM Tris-Hcl, pH 7.8
100mM MgCl2
100mM dithiothreitol (DTT)
10mM adenosine triphosphate (ATP)

10x Mutagenesis buffer
100mM Tris-acetate (pH 7.5)
100mM MgOAc
500mM KOAc (pH 7.5)

6% denaturing polyacrylamide gel.
40% acrylamide 19:1 bisacrylamide mix
250g urea
50ml 10xTBE
175ml DDW.
Stir until urea dissolves and store at 4°C.
Add 100µl TEMED and 100ml 25% ammonium persulphate per 100ml gel, mix and pour immediately.

100 ml formaldehyde/agarose gel
1 g agarose
20 ml 10x MOPS
62 ml DEPC treated water
boli by microwaving
cool to 60° and add 18 ml formaldehyde - pour immediately

RNA sample buffer
240µl formaldehyde
750µl deionised formaldehyde
50µl 10x MOPS running buffer
store at -20°C

RNA Loading Buffer
50% glycerol
1 mM EDTA
0.25% bromophenol blue
0.25% xylene cyanol

Northern Prehybridisation solution
2.5 ml 20x SSPE
1 ml 50x Denhart's solution
0.1 ml (10 mg/ml) Calf thymus DNA
5 ml formamide
to 10 ml with DEPC-treated DDW

Northern Hybridisation solution
2.5 ml 20x SSPE
1 ml Denhart's solution
0.1 ml Calf thymus DNA
5 ml 10% dextran sulphate in formamide to 10 ml with DEPC-treated DDW

Deionised Formamide.
5g Mixed bed resin per 100ml formamide. Stir 1 hour. Add more resin if required. Filter. Store at -20°C.

20x SSC
175.3g NaCl
88.2g Na citrate
to 1 litre with DDW (pH 7.0)

20xSSPE
174g NaCl
27.6g $NaH_2PO_4.H_2O$
7.4g EDTA
to 1 litre with DDW (pH 7.4)

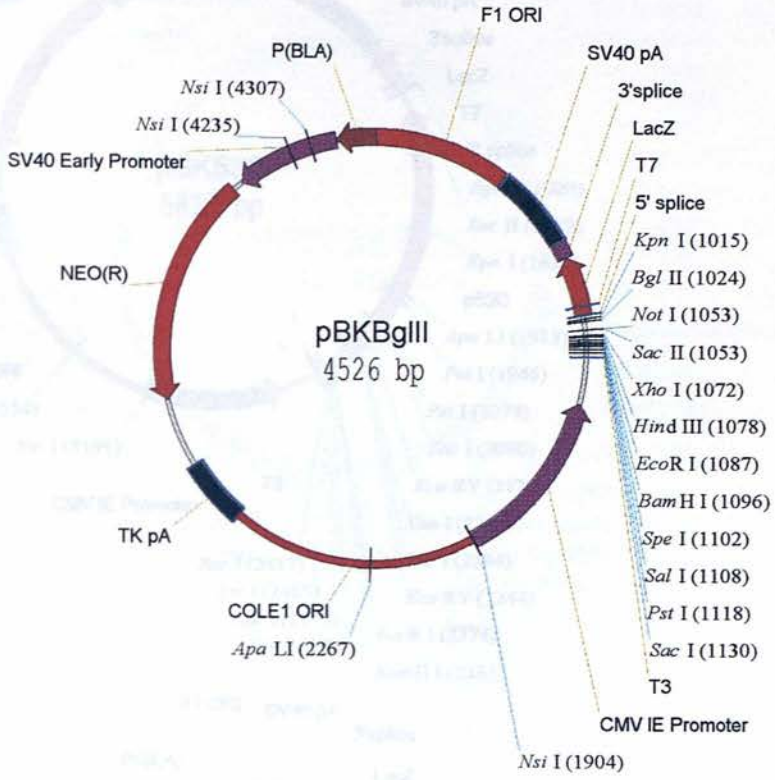
100x Denhardt's Solution.
20g polyvinyl pyrolidine
20g Ficoll
20g BSA
To 1 litre with DDW
Store at -20°C

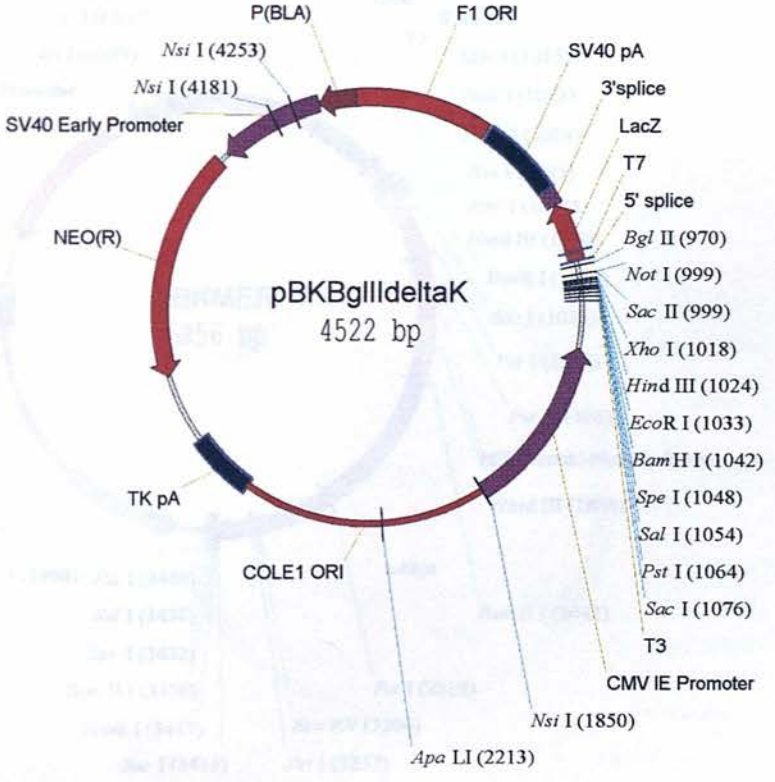
DNA-DNA Hybridisation Buffer.
5g Dextran sulphate in 25ml DDW, (65°C)
15ml 20xSSC
2.5ml 20% SDS
0.8ml 5mg/ml heat denatured salmon sperm DNA.

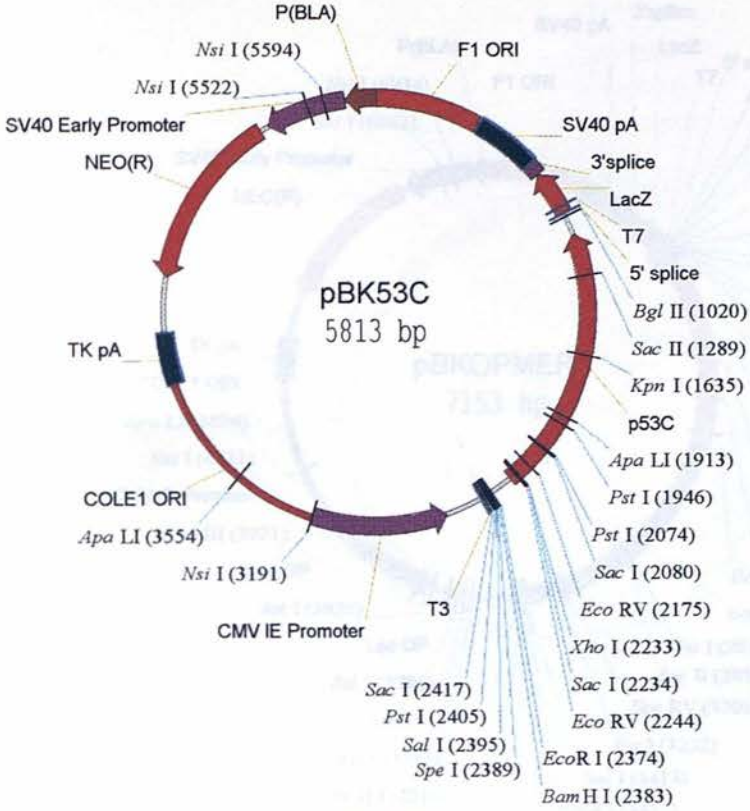
Appendix B: *E. coli* Strain Genotypes.

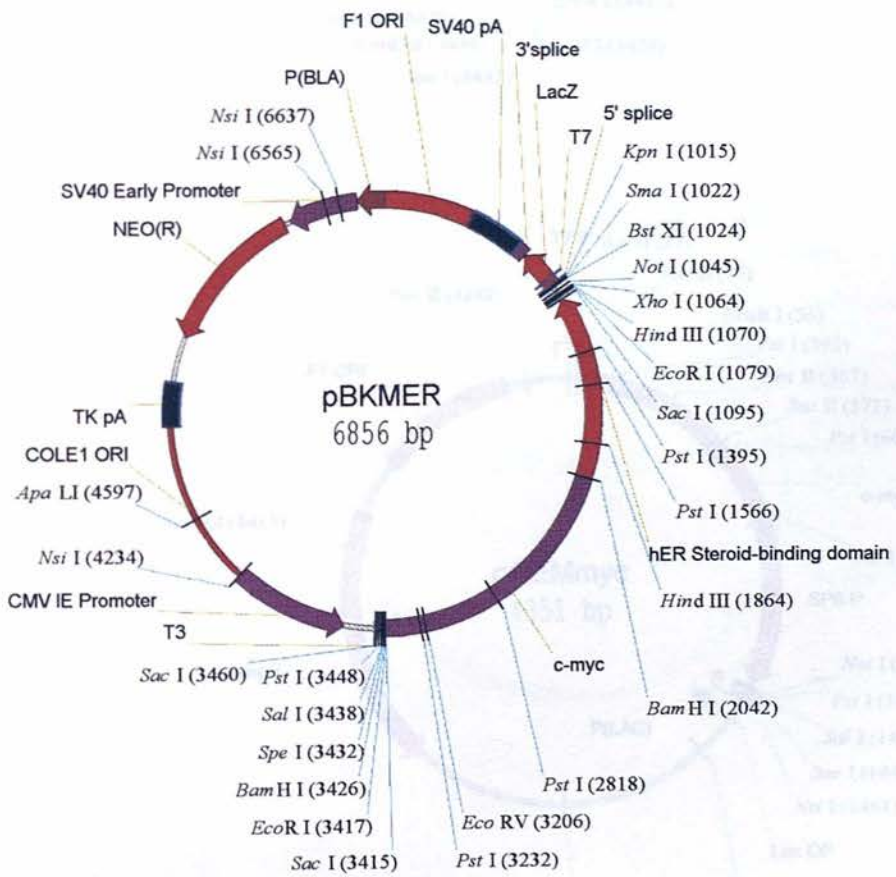
Strain	Genotype
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* {F' *proAB lacI*^{q}*ZΔM15* Tn10 (Tet^r)]^c
XL1BlueMR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*^{c}
XL1BlueMRF'	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn10 (Tet^r)]^c
XL*mut*S Kan^s	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac mutS::*Tn10 (Tet^r) [F' *proAB lacI*^{q}*ZΔM15* Tn5]
SCS110	*rpsL* (Str^r) *thr leu endA thi-1 lacY gaK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F' traD36 *proAB lacI*^{q}*ZΔM15*]

Appendix C: Additional Plasmid Maps.






pBK53C
5813 bp
P(BLA)
Nsi I (5594)
Nsi I (5522)
SV40 Early Promoter
NEO(R)
TK pA
COLE1 ORI
Apa LI (3554)
Nsi I (3191)
CMV IE Promoter
T3
Sac I (2417)
Pst I (2405)
Sal I (2395)
Spe I (2389)
EcoR I (2374)
BamH I (2383)
F1 ORI
SV40 pA
3'splice
LacZ
T7
5' splice
Bgl II (1020)
Sac II (1289)
Kpn I (1635)
p53C
Apa LI (1913)
Pst I (1946)
Pst I (2074)
Sac I (2080)
Eco RV (2175)
Xho I (2233)
Sac I (2234)
Eco RV (2244)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F1 ORI
SV40 pA
3'splice
LacZ
T7
5' splice
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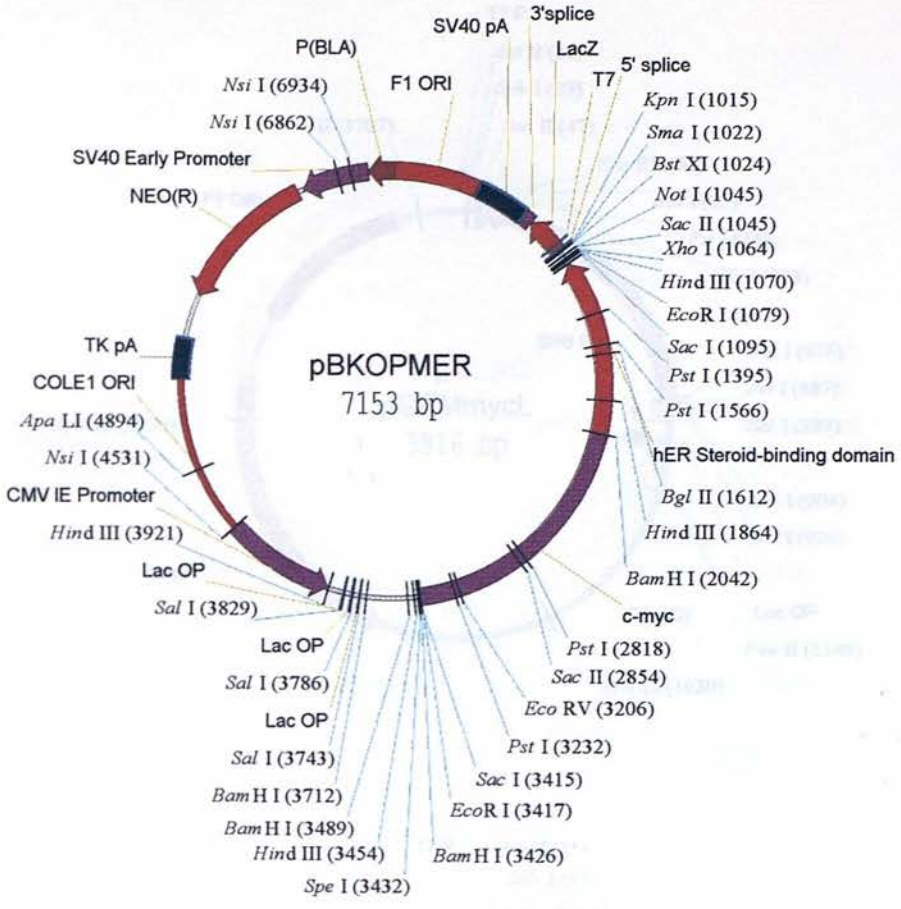
Nsi I (6565)
SV40 Early Promoter
NEO(R)
TK pA
COLE1 ORI
Apa LI (4597)
Nsi I (4234)
CMV IE Promoter
T3
Sac I (3460)
Pst I (3448)
Sal I (3438)
Spe I (3432)
BamH I (3426)
EcoR I (3417)
Sac I (3415)
Kpn I (1015)
Sma I (1022)
Bst XI (1024)
Not I (1045)
Xho I (1064)
Hind III (1070)
EcoR I (1079)
Sac I (1095)
Pst I (1395)
Pst I (1566)
hER Steroid-binding domain
Hind III (1864)
c-myc
BamH I (2042)
Pst I (2818)
Eco RV (3206)
Pst I (3232)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5' splice
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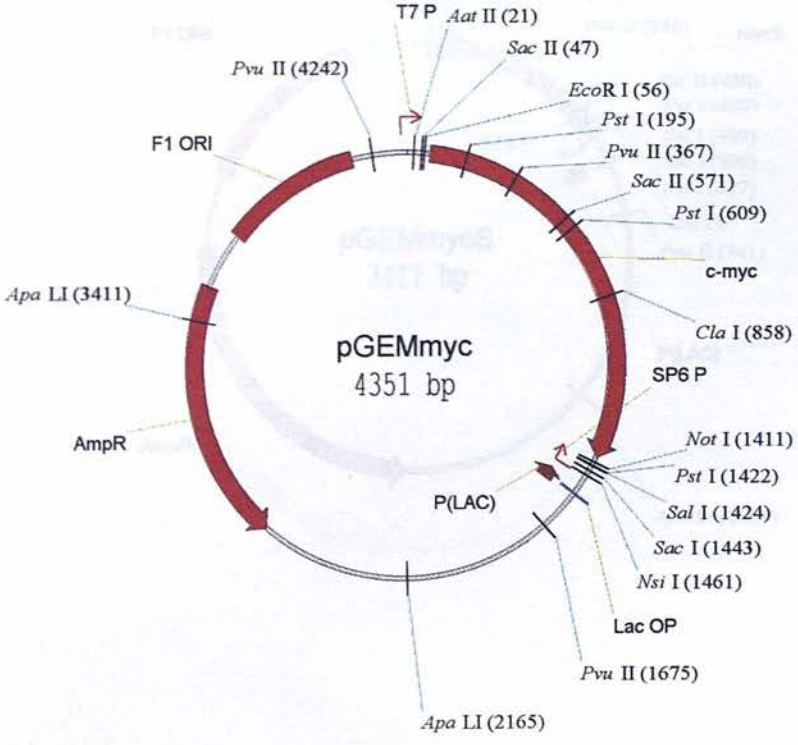
F1 ORI
Nsi I (6934)
Nsi I (6862)
SV40 Early Promoter
NEO(R)
TK pA
COLE1 ORI
Apa LI (4894)
Nsi I (4531)
CMV IE Promoter
Hind III (3921)
Lac OP
Sal I (3829)
Lac OP
Sal I (3786)
Lac OP
Sal I (3743)
Bam H I (3712)
Bam H I (3489)
Hind III (3454)
Spe I (3432)
Bam H I (3426)
Kpn I (1015)
Sma I (1022)
Bst XI (1024)
Not I (1045)
Sac II (1045)
Xho I (1064)
Hind III (1070)
EcoR I (1079)
Sac I (1095)
Pst I (1395)
Pst I (1566)
hER Steroid-binding domain
Bgl II (1612)
Hind III (1864)
Bam H I (2042)
c-myc
Pst I (2818)
Sac II (2854)
Eco RV (3206)
Pst I (3232)
Sac I (3415)
EcoR I (3417)


pGEMmyc
4351 bp
T7 P
Aat II (21)
Sac II (47)
Pvu II (4242)
EcoR I (56)
Pst I (195)
F1 ORI
Pvu II (367)
Sac II (571)
Pst I (609)
c-myc
Apa LI (3411)
Cla I (858)
SP6 P
AmpR
Not I (1411)
Pst I (1422)
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Sal I (1424)
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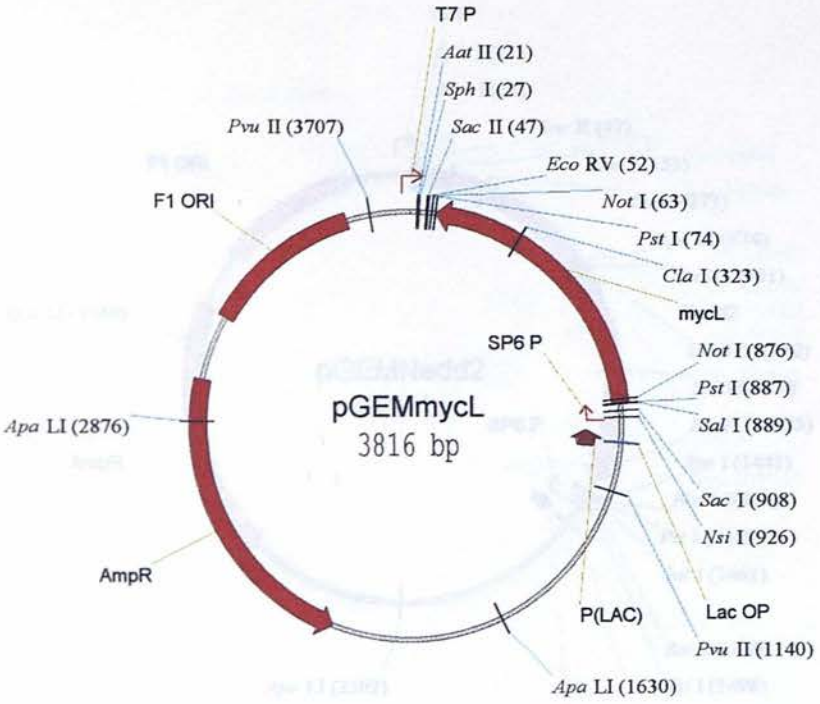
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Pst I (74)
Cla I (323)
mycL
SP6 P
Not I (876)
Pst I (887)
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3816 bp
Apa LI (2876)
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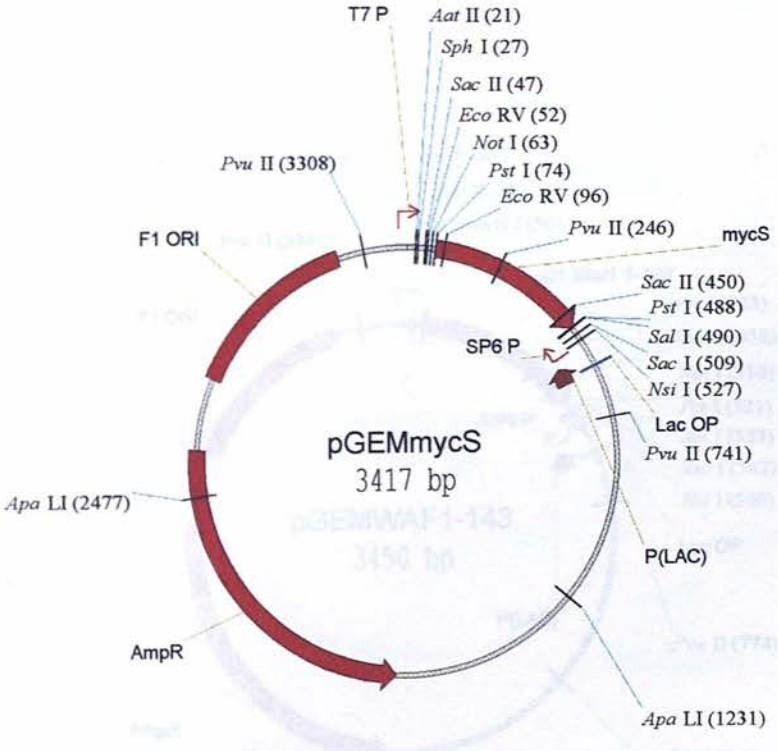
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Eco RV (96)
Pvu II (246)
mycS
F1 ORI
Sac II (450)
Pst I (488)
Sal I (490)
SP6 P
Sac I (509)
Nsi I (527)
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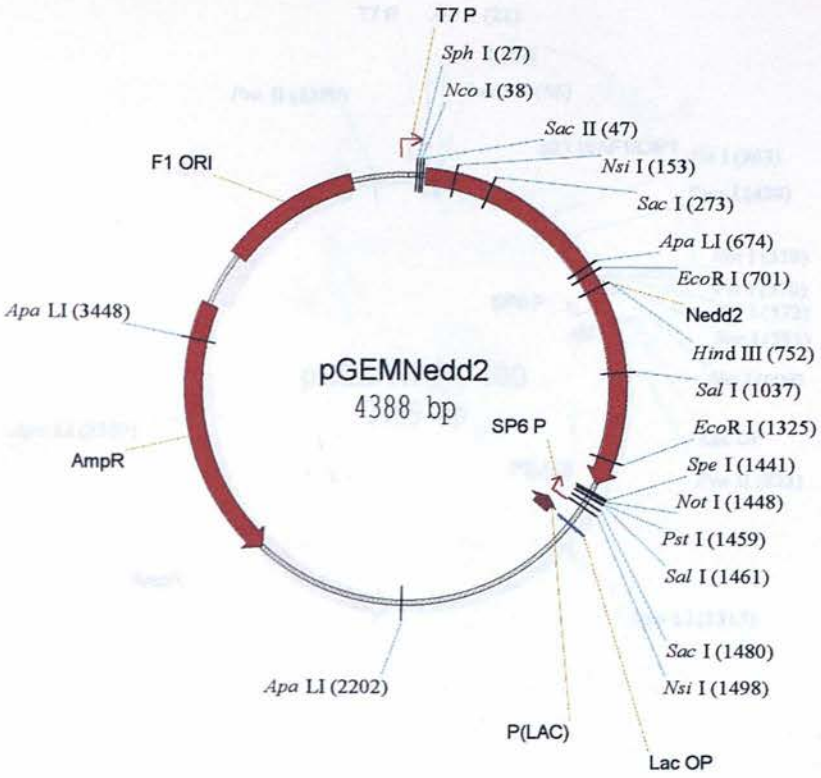
AmpR
Apa LI (1231)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Sac I (273)
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Nedd2
Hind III (752)
Sal I (1037)
EcoR I (1325)
Spe I (1441)
Not I (1448)
Pst I (1459)
Sal I (1461)
Sac I (1480)
Nsi I (1498)
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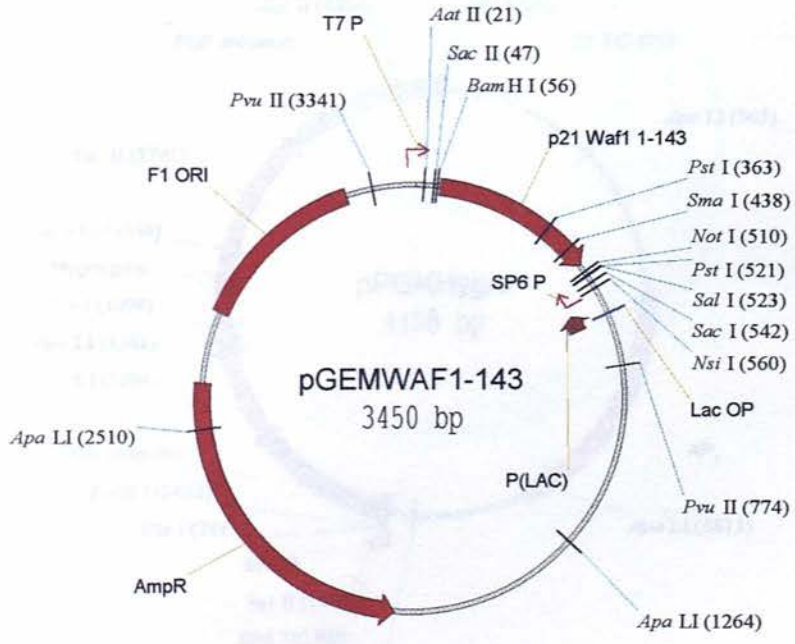
T7 P
Aat II (21)
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Bam H I (56)
Pvu II (3341)
p21 Waf1 1-143
Pst I (363)
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Not I (510)
Pst I (521)
Sal I (523)
Sac I (542)
Nsi I (560)
Lac OP
SP6 P
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Pvu II (774)
Apa LI (1264)
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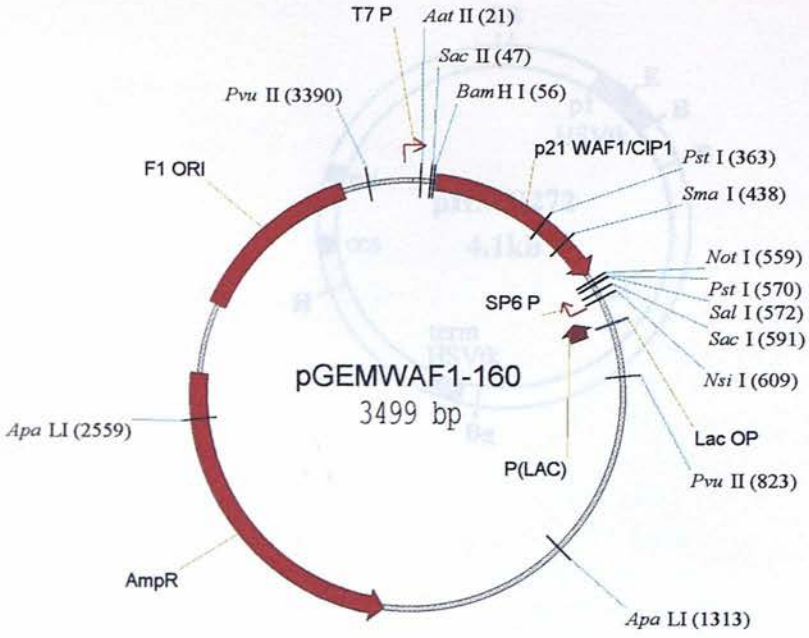
pGEMWAF1-143
3450 bp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p21 WAF1/CIP1
Pst I (363)
Sma I (438)
Not I (559)
Pst I (570)
Sal I (572)
Sac I (591)
SP6 P
pGEMWAF1-160
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Apa LI (2559)
Lac OP
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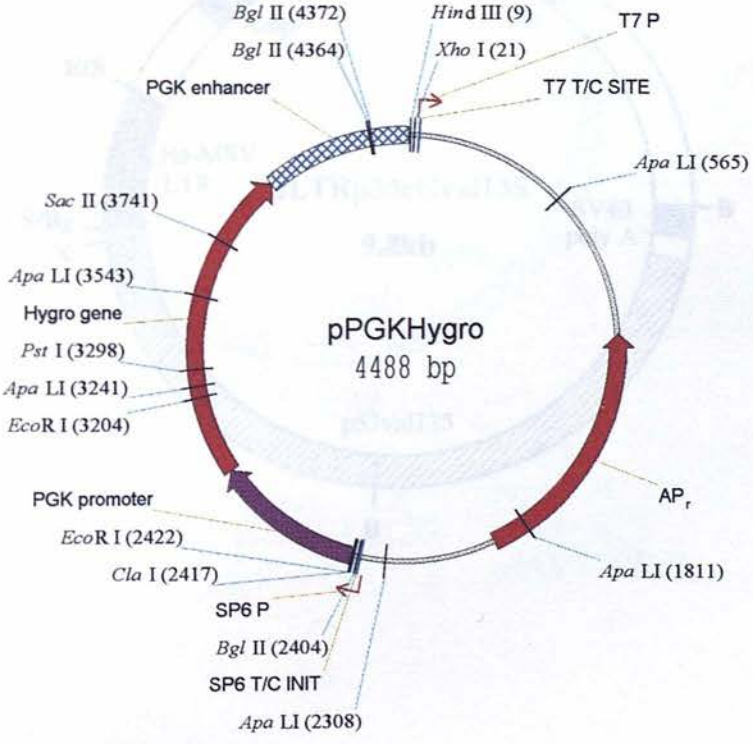
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Hind III (9)
T7 P
Bgl II (4364)
Xho I (21)
PGK enhancer
T7 T/C SITE
Apa LI (565)
Sac II (3741)
Apa LI (3543)
Hygro gene
pPGKHygro
4488 bp
Pst I (3298)
Apa LI (3241)
EcoR I (3204)
PGK promoter
AP r
EcoR I (2422)
Cla I (2417)
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SP6 P
Bgl II (2404)
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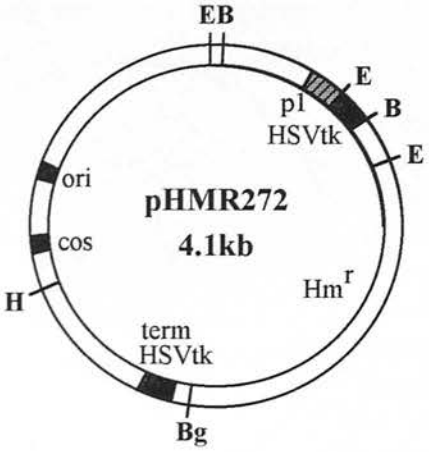
Apa LI (2308)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E
pI
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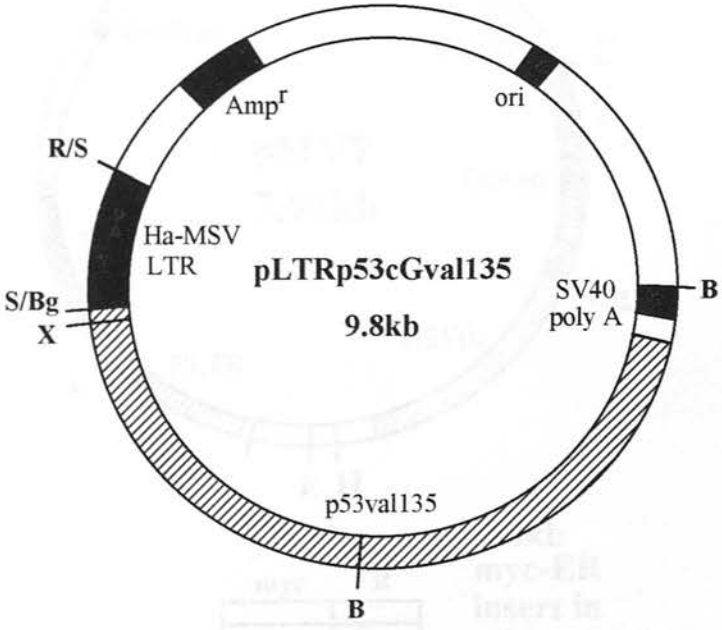
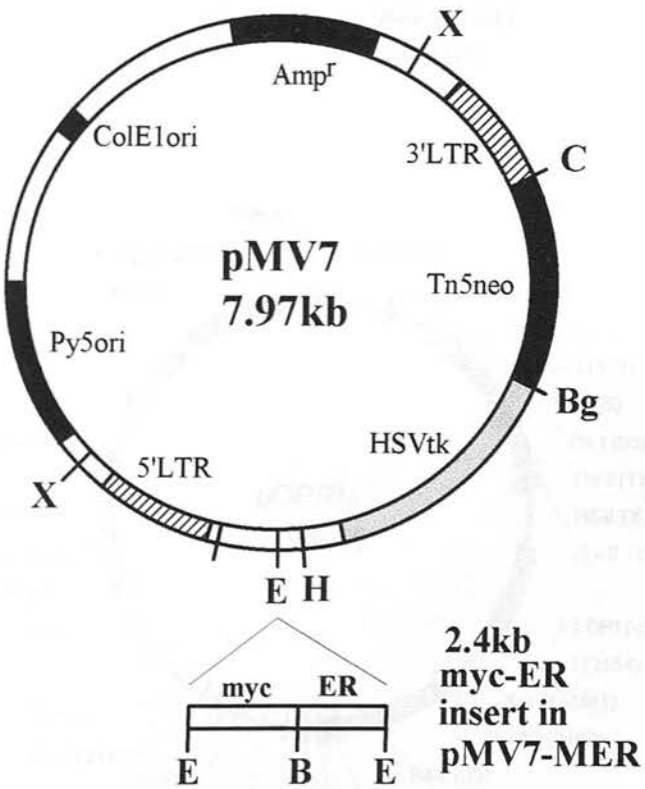
B
E
ori
pHMR272
4.1kb
cos
H
Hm r
term
HSVtk
Bg


Amp r
ori
R/S
Ha-MSV
LTR
pLTRp53cGval135
9.8kb
S/Bg
X
SV40
poly A
B
p53val135
B


X
Amp^r
ColE1ori
3'LTR
C
pMV7
7.97kb
Tn5neo
Py5ori
Bg
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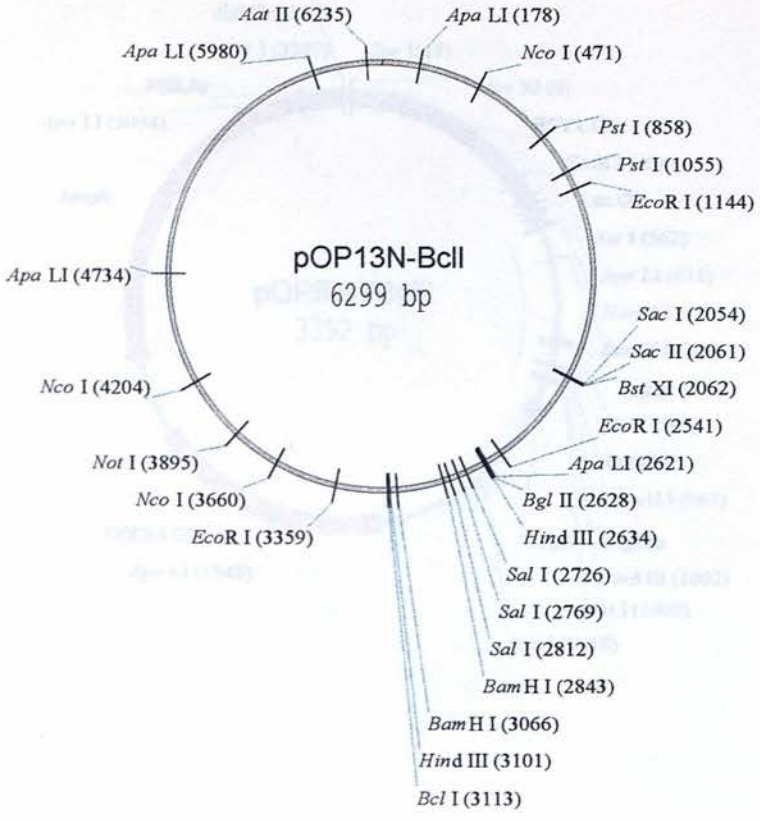
X
E H
myc
ER
E
B
E
2.4kb
myc-ER
insert in
pMV7-MER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Pst I (858)
Pst I (1055)
EcoR I (1144)
Apa LI (4734)
Sac I (2054)
Sac II (2061)
Nco I (4204)
Bst XI (2062)
EcoR I (2541)
Not I (3895)
Apa LI (2621)
Nco I (3660)
Bgl II (2628)
EcoR I (3359)
Hind III (2634)
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Sal I (2812)
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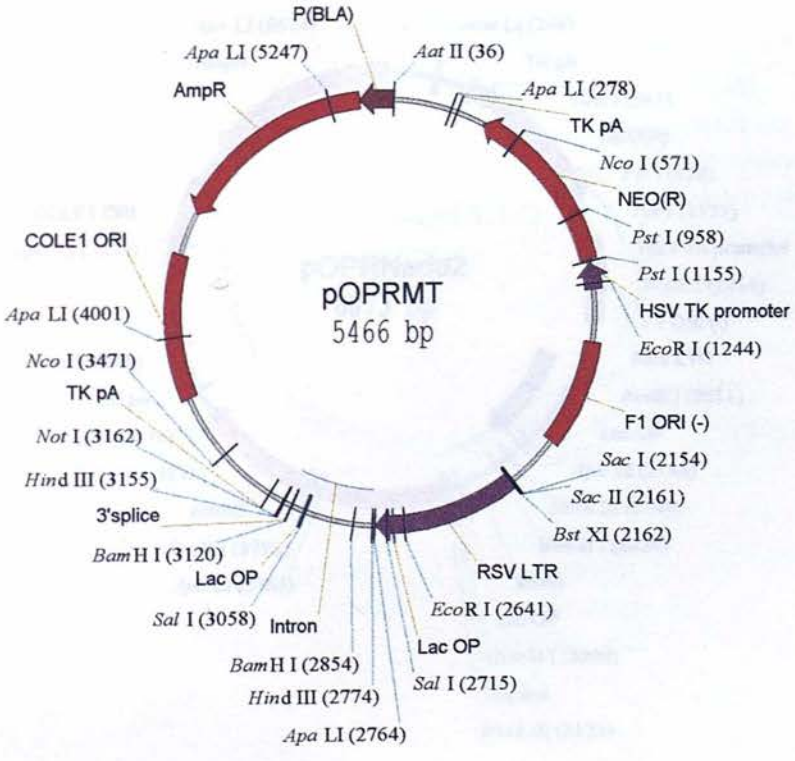
Hind III (3101)
Bcl I (3113)


pOPRMT
5466 bp
P(BLA)
Apa LI (5247)
Aat II (36)
AmpR
Apa LI (278)
TK pA
Nco I (571)
NEO(R)
COLE1 ORI
Pst I (958)
Pst I (1155)
Apa LI (4001)
HSV TK promoter
EcoR I (1244)
Nco I (3471)
TK pA
F1 ORI (-)
Not I (3162)
Sac I (2154)
Hind III (3155)
Sac II (2161)
3'splice
Bst XI (2162)
Bam H I (3120)
RSV LTR
Lac OP
EcoR I (2641)
Sal I (3058)
Intron
Lac OP
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Sal I (2715)
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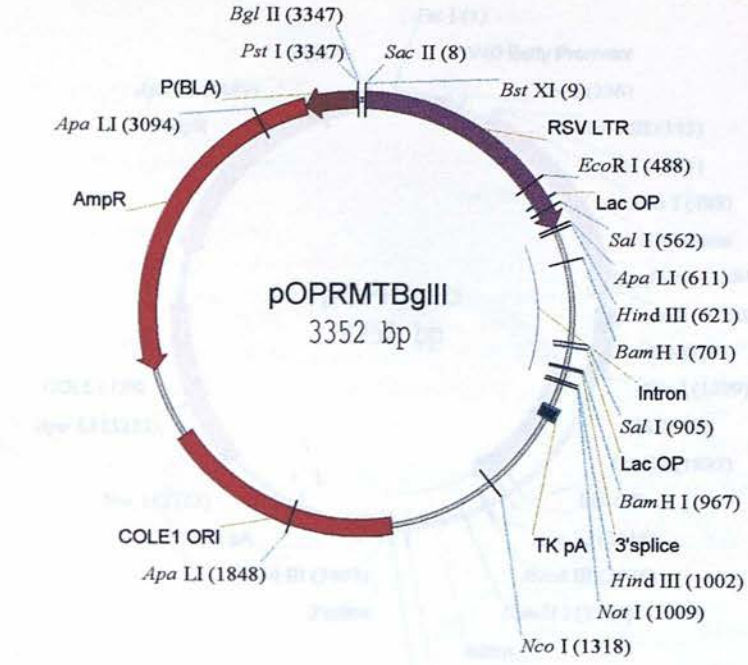
Apa LI (2764)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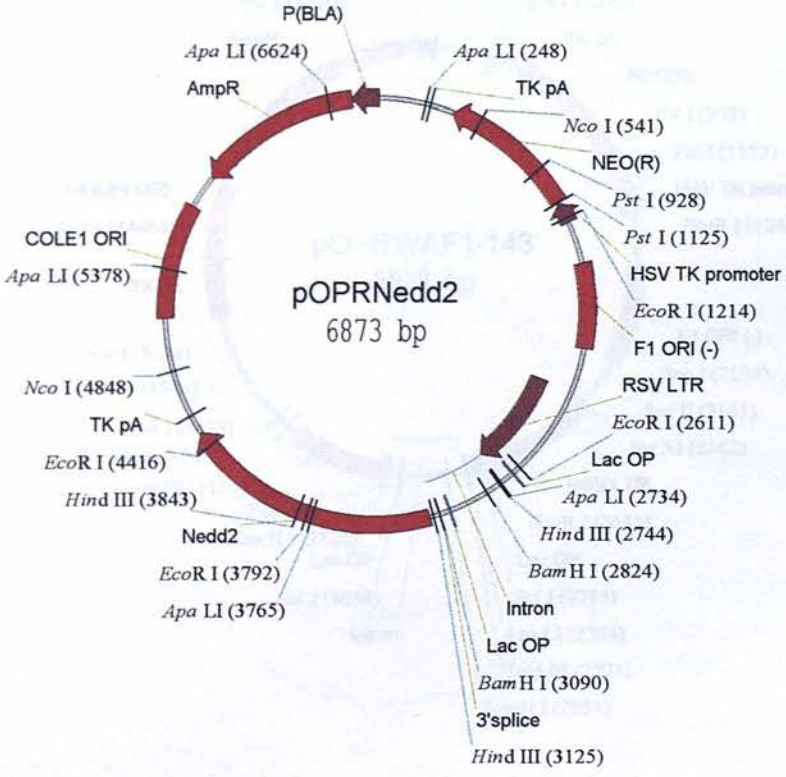
Pst I (3347)
Sac II (8)
Bst XI (9)
P(BLA)
Apa LI (3094)
RSV LTR
EcoR I (488)
AmpR
Lac OP
Sal I (562)
Apa LI (611)
Hind III (621)
Bam H I (701)
Intron
Sal I (905)
Lac OP
Bam H I (967)
COLE1 ORI
TK pA
3'splice
Apa LI (1848)
Hind III (1002)
Not I (1009)
Nco I (1318)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AmpR
TK pA
Nco I (541)
NEO(R)
Pst I (928)
Pst I (1125)
COLE1 ORI
Apa LI (5378)
HSV TK promoter
EcoR I (1214)
F1 ORI (-)
Nco I (4848)
RSV LTR
TK pA
EcoR I (2611)
EcoR I (4416)
Lac OP
Hind III (3843)
Apa LI (2734)
Nedd2
Hind III (2744)
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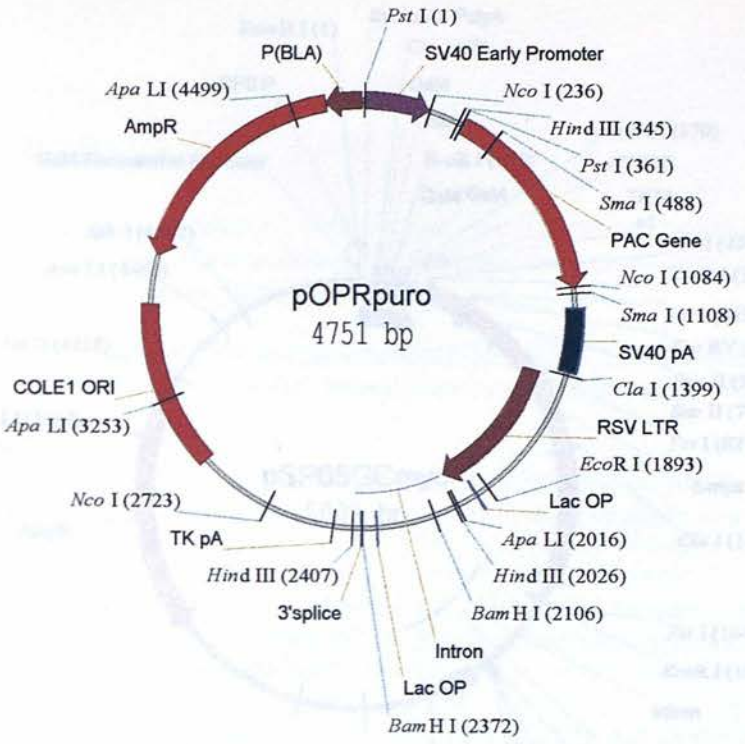
Intron
Lac OP
Bam H I (3090)
3'splice
Hind III (3125)


pOPRpuro
4751 bp
Pst I (1)
P(BLA)
SV40 Early Promoter
Apa LI (4499)
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Hind III (345)
Pst I (361)
Sma I (488)
PAC Gene
Nco I (1084)
Sma I (1108)
SV40 pA
COLE1 ORI
Cla I (1399)
Apa LI (3253)
RSV LTR
EcoR I (1893)
Nco I (2723)
Lac OP
TK pA
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Hind III (2026)
3'splice
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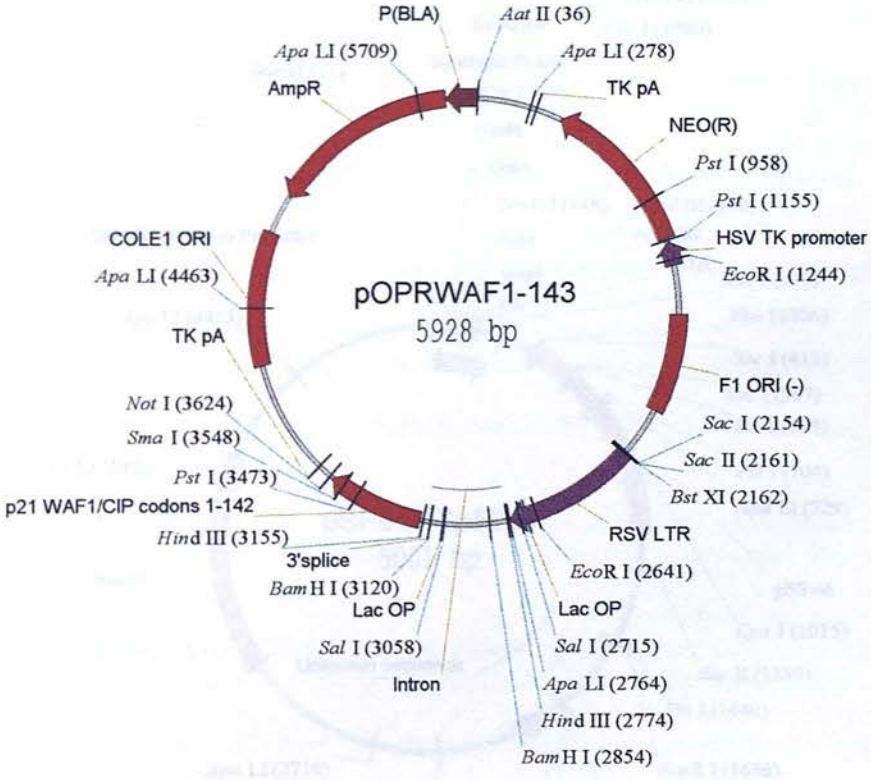
Lac OP
Bam H I (2372)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TK pA
NEO(R)
Pst I (958)
Pst I (1155)
HSV TK promoter
COLE1 ORI
EcoR I (1244)
Apa LI (4463)
TK pA
F1 ORI (-)
Not I (3624)
Sac I (2154)
Sma I (3548)
Sac II (2161)
Pst I (3473)
Bst XI (2162)
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RSV LTR
Hind III (3155)
3'splice
EcoR I (2641)
Bam H I (3120)
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Lac OP
Sal I (3058)
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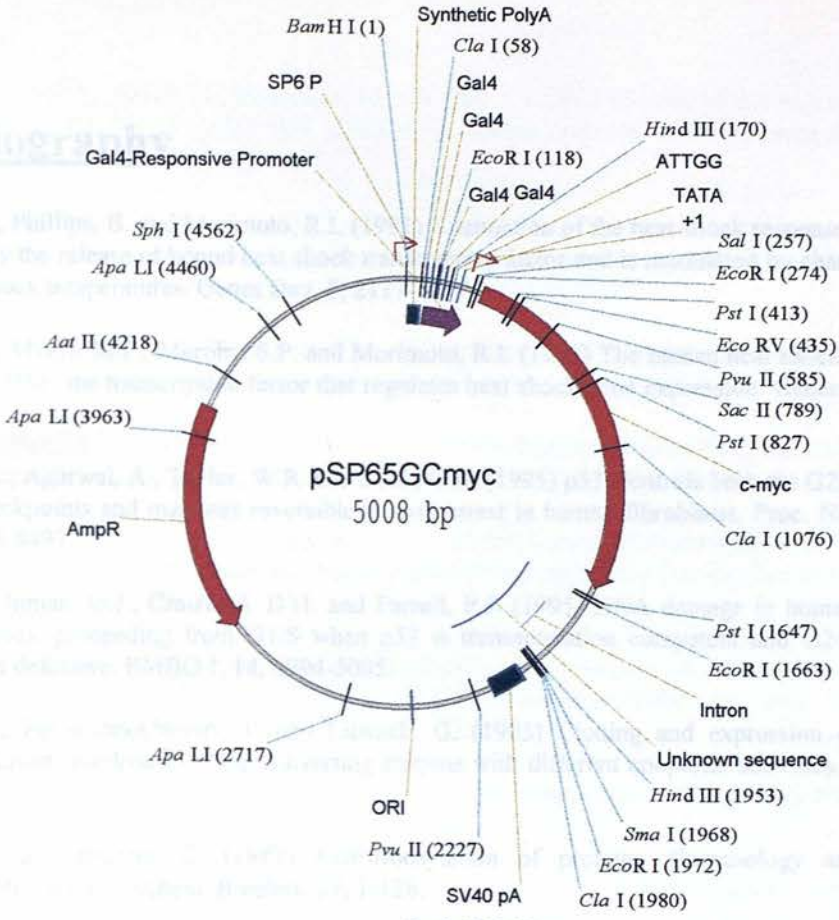
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Synthetic PolyA
Cla I (58)
SP6 P
Gal4
Gal4
Hind III (170)
Gal4-Responsive Promoter
EcoR I (118)
ATTGG
Gal4 Gal4
TATA
+1
Sph I (4562)
Sal I (257)
Apa LI (4460)
EcoR I (274)
Pst I (413)
Aat II (4218)
Eco RV (435)
Pvu II (585)
Apa LI (3963)
Sac II (789)
Pst I (827)
pSP65GCmyc
5008 bp
c-myc
AmpR
Cla I (1076)
Pst I (1647)
EcoR I (1663)
Intron
Apa LI (2717)
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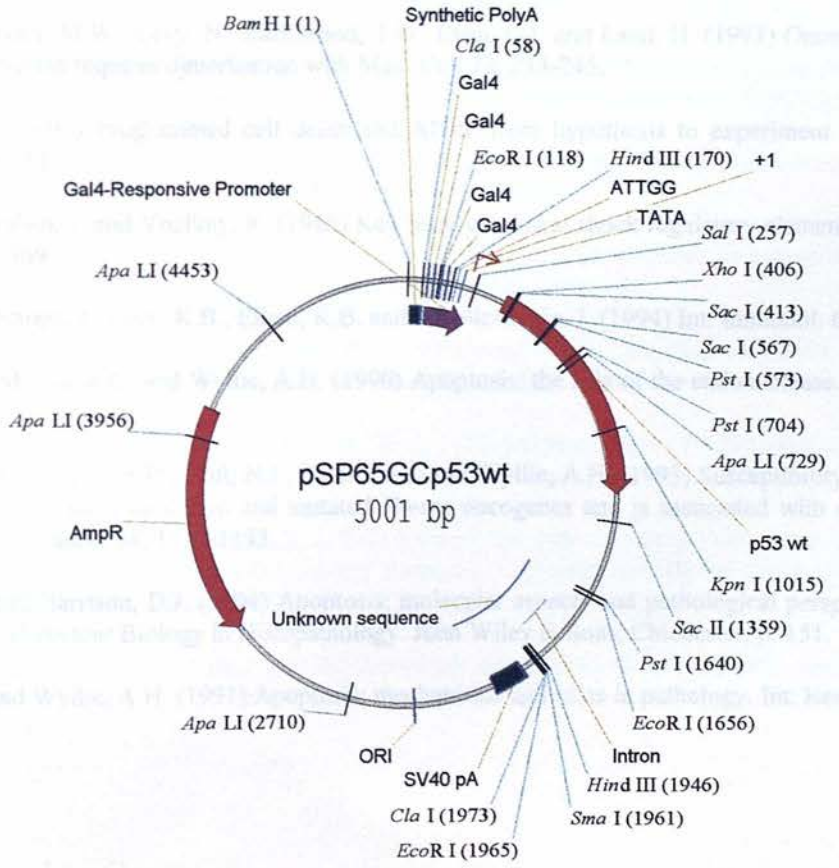
Hind III (1953)
ORI
Sma I (1968)
Pvu II (2227)
EcoR I (1972)
SV40 pA
Cla I (1980)


Bam H I (1)
Synthetic PolyA
Cla I (58)
Gal4
Gal4
EcoR I (118)
Hind III (170)
+1
Gal4-Responsive Promoter
Gal4
ATTGG
Gal4
TATA
Sal I (257)
Apa LI (4453)
Xho I (406)
Sac I (413)
Sac I (567)
Pst I (573)
Apa LI (3956)
Pst I (704)
Apa LI (729)
pSP65GCp53wt
5001 bp
AmpR
p53 wt
Kpn I (1015)
Unknown sequence
Sac II (1359)
Pst I (1640)
Apa LI (2710)
EcoR I (1656)
ORI
Intron
SV40 pA
Hind III (1946)
Cla I (1973)
Sma I (1961)
EcoR I (1965)

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p53-independent death and p53-induced protection against apoptosis in fibroblasts treated with chemotherapeutic drugs

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Summary Many recent studies have implicated p53 in the cellular response to injury and induction of cell death by apoptosis. In a rat embryonal fibroblast cell line transformed with c-Ha-*ras* and a mutant temperature-sensitive p53 (val135), cells were G_1 arrested at the permissive temperature of 32°C when overexpressed p53 was in wild-type conformation. In this state cells were resistant to apoptosis induced by etoposide (at up to 50 μM) or bleomycin (15 μU ml^{-1}). Cells at 37°C with overexpressed p53 in mutant conformation were freed from this growth arrest, continued proliferating and showed dose-dependent increases in apoptosis. This death is independent of wild-type p53 function. Control cells containing a non-temperature-sensitive mutant p53 (phe132) were sensitive to both etoposide and bleomycin after 24 h at 32°C and 37°C, indicating that the results are not simply due to temperature effects on pharmacokinetics or DNA damage. Our data show that induction of a stable p53-mediated growth arrest renders these cells much less likely to undergo apoptosis in response to certain anti-cancer drugs, and we conclude that the regulatory role of p53 in apoptosis is influenced by the particular cellular context in which this gene is expressed.

Keywords: p53; *ras*; apoptosis; etoposide; bleomycin; cell cycle; fibroblasts

Many tumours are resistant to chemotherapy, either intrinsically or following an initial partial response. A number of pharmacokinetic explanations may account for this, including overexpression of the multidrug resistance gene *mdr*1, overexpression of drug detoxication enzymes, or alteration of the drug target, for example topoisomerase II isoform. However despite intensive study of drug–target interactions, and drug metabolism, it is clear that in many instances drug resistance is associated with a failure of induction of apoptosis, even after an appropriate triggering event. Since many anti-cancer drugs and ionising radiation damage DNA, the response of the cell in recognising injury and proceeding to repair or apoptosis is of paramount importance (Hickman, 1992; Harrison, 1995).

Entry to apoptosis is regulated by a number of genes (see Bellamy *et al.*, 1995 for general review), each of which may show abnormal expression or function in cancer. In Rat-1 fibroblasts cell cycle arrest or serum deprivation in the presence of constitutive expression of the c-*myc* oncogene can cause apoptosis (Evan *et al.*, 1992). By contrast, overexpression of *bcl*-2 directly inhibits apoptosis in both normal and neoplastic cells (Hockenberry *et al.*, 1990; Sentman *et al.*, 1991; Miyashita and Reed, 1992, 1993; Veis *et al.*, 1993) and prevents c-*myc*-driven apoptosis (Wagner *et al.*, 1993). More recently evidence has accumulated implicating the tumour-suppressor gene p53 in an injury-response pathway leading to apoptosis. Thymocytes and myeloid progenitor cells from p53 knockout mice, fail to undergo *induced* apoptosis in the absence of a wild-type p53 allele following etoposide or ionising radiation treatment but not apoptosis associated with ageing *in vitro* or non-clastogenic insults such as dexamethasone treatment. (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993*a*). Furthermore overexpression of wild-type p53 in a variety of cancer-derived cell lines such as M1 myeloid leukaemia (Yonish-Rouach *et al.*, 1991), murine erythroleukaemia (Ryan *et al.*, 1993) and HT29 colon carcinoma (Shaw *et al.*, 1992) resulted in an increase in *spontaneous* apoptosis.

By contrast, studies of p53 null fibroblasts grown in primary culture have failed to detect alteration in cell survival characteristics after DNA damage as compared with normal primary fibroblasts (Slichenmeyer *et al.*, 1993). In the latter experiments, cells were isogenic apart from p53 status. This suggests that other factors, including cell lineage and expression of oncogenes may modulate the effects of p53 on cellular physiology. In both experimental and human tumorigenesis p53 inactivation is believed to be a late event and is therefore superimposed on a series of progressive genetic abnormalities, such as activation of *ras* oncogenes (Fearon and Vogelstein, 1990).

In this study we have used a rat embryonal fibroblast line (Clone 6) transformed with activated Ha-*ras* and a temperature-sensitive p53 mutant as a model of the role of p53 in anti-cancer drug therapy in the presence of other genetic alterations. We report that induction by wild-type p53 of a G_1 arrest protects Clone 6 cells from apoptosis caused by the anti-cancer drugs etoposide and bleomycin. Our data imply that wild-type p53 provides a mechanism of resistance of cells to chemotherapy but by allowing continued proliferation p53 mutations may nonetheless contribute to the development of drug resistance.

Materials and methods

Clone 6 cells and RcGphe132.4 cells

Clone 6 cells are rat embryonic fibroblasts constitutively expressing a human mutationally activated c-Ha-*ras*1 gene and a murine, temperature-sensitive p53 mutant, p53val135. At the permissive temperature of 32°C the p53 protein is found predominantly in wild-type configuration but at 37.5°C it adopts mutant conformation and function (Michalovitz *et al.*, 1990). RcGphe132.4 cells contain a temperature-stable p53phe132 mutation in addition to activated c-Ha-*ras*1. The level of p53 expression in these two cell lines is similar. All manipulation of cell lines and counting was performed at the selected temperature to minimise the risk of inadvertent p53 conformational shifts.

Cell culture

Cells were plated in duplicate flasks at a density of 2×10^4 cells cm^{-2} in Glasgow modified Eagle's medium (GMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

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Quantitation of cell number and apoptosis

Reference points (three per flask) were used to count directly the number of cells in × 100 field using a 10 × 10 graticule. This permitted sequential counts at 20, 28 and 42 h after plating at 32°C or 37°C. Apoptotic cells adherent to the monolayer were counted at each time point as well as cell number. The apoptotic cells were recognised by virtue of their spherical, highly refractile appearance under phase contrast. These cells showed the classical appearances of apoptosis and were confirmed by electron microscopy and acridine orange fluorescence microscopy (Arends and Harrison, 1994).

Effects of bleomycin and etoposide

Twenty four hours after plating at 37°C cells were either moved to a 32°C incubator or maintained at 37°C for a further 16 h. Etoposide (10, 50 μM) or bleomycin sulphate (15 μU ml^{-1}: 1 U = 1 mg bleomycin A2) were added for 1 h and then washed with phosphate buffered saline (PBS). Controls were performed using equal concentrations of dimethyl sulphoxide (DMSO) or PBS vehicles.

The number of viable and apoptotic cells was counted at intervals up to 50 h following drug treatment. The mean number of apoptotic bodies per field was expressed as a percentage of the mean adherent cell number ('percentage apoptosis'). RcGphe132.4 cells were counted 24 h after drug treatment.

Cell cycle analysis

Nuclei were isolated and stained with propidium iodide (Vindelov *et al.*, 1983), and 1×10^4 cells were analysed on an EPICS CS flow cytometry (Coulter). Histogram analysis was performed using the Easy 2 Software. No doublets were seen. Bromodeoxyuridine incorporation analysis was carried out using the Amersham Cell Proliferation Kit (cat no. RPN20).

Results

Clone 6 cells are growth arrested at 32°C

Exponentially growing cells were shifted to a 32°C incubator. In three independent experiments cells ceased to show increase in cell number at 32°C (Figure 1). There was no increase in apoptosis in the presence of p53 with wild-type configuration (see Figure 2 controls). At 37°C, with mutant conformation p53 there was a 3-fold increase in cell number over the same period, confirming the original observations of Michalovitz *et al.* (1990). DNA flow cytometry showed both diploid and tetraploid peaks at permissive and non-permissive temperatures. At 32°C there was in increase in the diploid $G_{0/1}$ peak (Figure 3), and cells did not take up bromodeoxyuridine consistent with this state (data not shown). This growth arrest was reversible by transferring cells to 37°C, even after 2 weeks, or more. By contrast RcGphe132.4 cells continued to grow at a slightly reduced rate at 32°C in keeping with the previous observations of Michalovitz *et al.* (1990).

Clone 6 cells with wild type p53 are resistant to both etoposide and bleomycin

At 37°C, in the presence of mutant conformation p53, there was a progressive increase in apoptosis starting 6–10 h after pulsing with drug (Figure 2). The increase was dose dependent: etoposide at 10 μM induced a maximum of 6% apoptosis whereas at 50 μM the maximum was greater than 30% apoptosis (Figure 4). By contrast, cells maintained at 32°C with p53 in the wild-type conformation showed no increase in percentage apoptosis, nor in cell number (Figures 2 and 4). Treatment with bleomycin showed similar effects (Figure 5).

RcGphe132.4 cells are sensitive to apoptosis induced by etoposide and bleomycin at 32°C and 37°C. We considered the possibility that these differences in cell proliferation and apoptosis in response to DNA damage might be due simply to altered pharmacokinetics at the different temperatures. The RcGphe132.4 cell line was derived from the same parental stock as Clone 6, but contains a temperature-insensitive mutant p53; hence in this cell line wild-type p53 is excluded from function at both 32°C and 37°C. At 37°C Clone 6 and RcGphe132.4 cells show closely similar entry into apoptosis: 24 h after treatment with 50 μM etoposide the incidences were 18.0% and 19.4% respectively. In contrast, at 32°C the incidence of apoptosis in RcGphe132.4 cells was 9.5%, but had fallen to less than 2% in Clone 6 cells. Very similar results were obtained following treatment with bleomycin. At 37°C incidence of apoptosis in Clone 6 cells was 19.8%, but fell to less than 3% at 32°C. In contrast, RcGphe132.4 cells

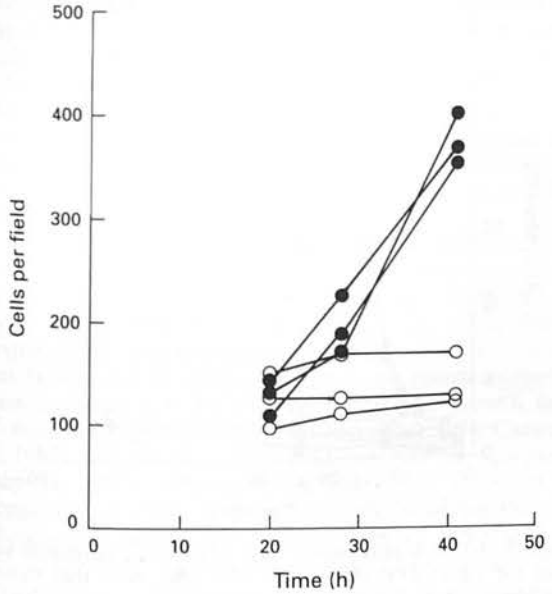

Figure 1 Growth properties of Clone 6 cells at 37°C (●) and at 32°C (○). Each point represents the mean number of cells per field ($n = 3$) in one flask at each time point. Note that the cells incubated at 32°C do not increase in number consistent with a wt p53-induced growth arrest.

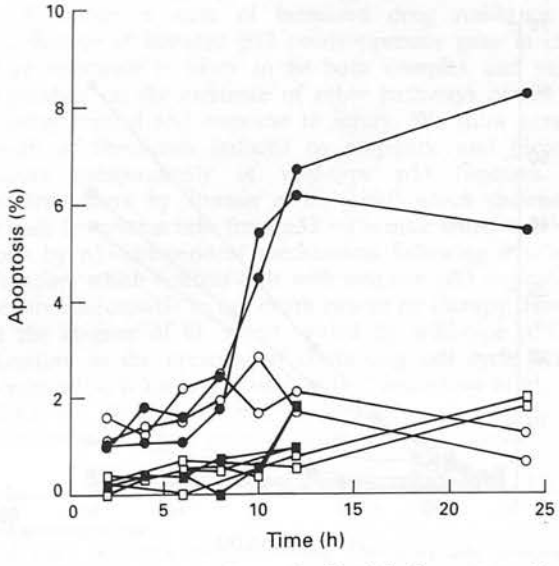

Figure 2 Treatment of Clone 6 cells with 10 μM etoposide at 37°C (●) for 1 h results in substantial apoptosis, where as treated cells at 32°C (■) and untreated controls (unfilled symbols) do not show this increase. Note the latent period during induction of apoptosis at 37°C. Each line represents a separate experiment (performed in triplicate and expressed as a mean, for low values the range was less than 0.6% and for higher value the range was up to 2%).

showed 10.3% apoptosis at 37°C and 8.9% at 32°C. Thus the profound inhibition of apoptosis in Clone 6 cells at 32°C is dependent upon the altered configuration of p53 to wild-type and is not explicable solely on the basis of temperature effects on pharmacokinetics.

Discussion

Expression of wt p53 has been shown to induce apoptosis in some cell types (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Ryan *et al.*, 1993), G_1 arrest and survival in others (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992). In addition, wt p53 has been shown to be an essential intermediate in a signal transduction pathway between the effects of DNA damaging agents (DNA strand breaks) and either apoptosis or G_1 arrest (Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993*a*). In this way p53 seems to play a critical role in deleting certain cell types that have sustained DNA damage e.g. thymocytes (Clarke *et al.*, 1993), lymphocytes (Gottlieb *et al.*, 1994; Howie *et al.*,

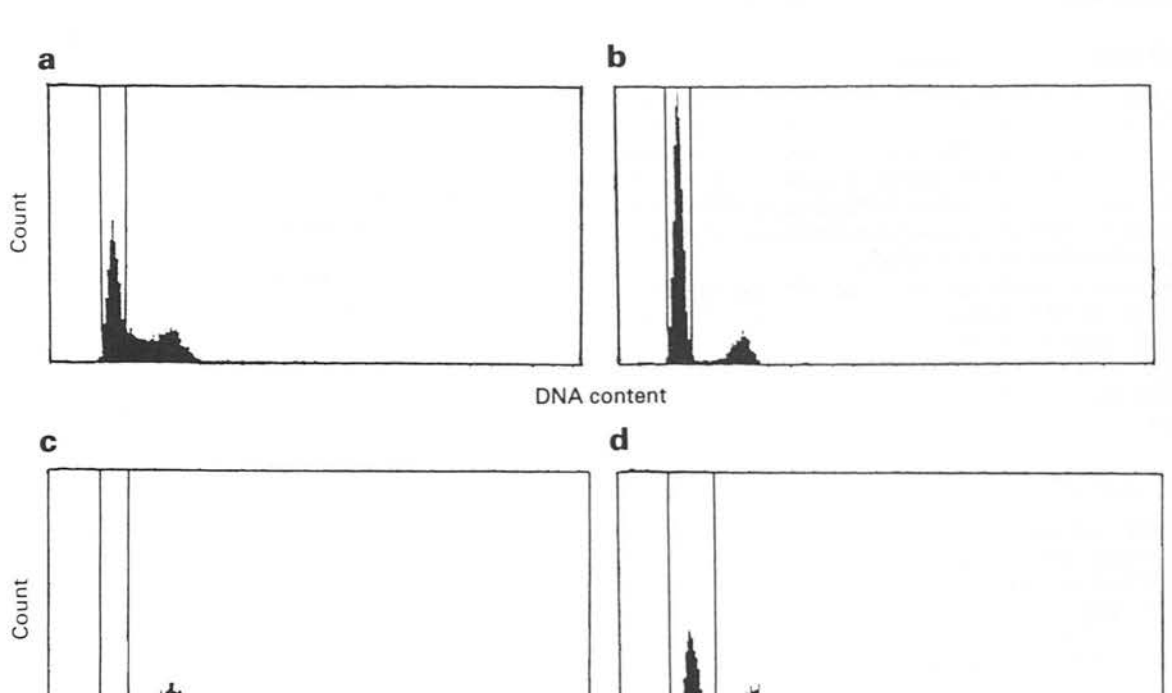

Figure 3 Cell cycle analysis of Clone 6 cells. (**a**) Exponentially growing cells at 37°C, untreated (G_0/G_1 fraction: 46.56%). (**b**) Following incubation at 32°C for 24 h, the G_0/G_1 peak is enlarged (72.51%) and there is a marked decrease in the proportion of cells between the G_0/G_1 and G_2/M peaks (S-phase). (**c**) At 37°C, 24 h after etoposide treatment (50 μM) cells accumulated in G_2/M with only 10.18% of cells occupying the G_0/G_1 position. (**d**) At 32°C, 24 h after treatment with 50 μM etoposide (G_0/G_1 fraction: 38.51%). Abcissa; DNA content (propidium iodide fluorescence).

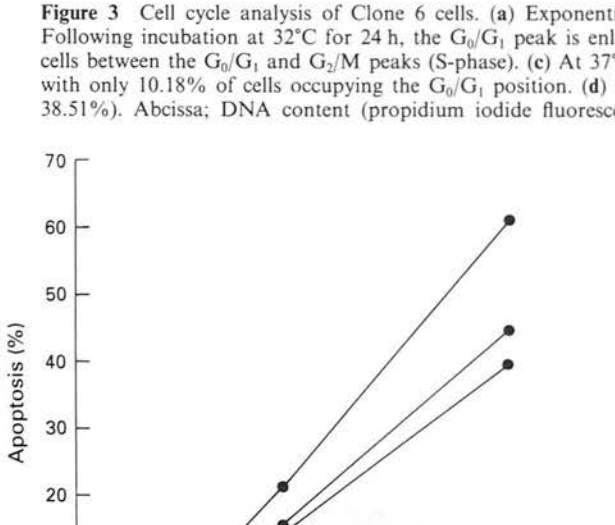

Figure 4 Treatment of Clone 6 cells with 50 μM etoposide for 1 h induces substantial apoptosis when cells are incubated at 37°C (●). At 32°C treated cells (■) and in control cells treated with an equivalent volume of DMSO vehicle (unfilled symbols) do not show an increase in percentage apoptosis. Each line represents a separate experiment (performed in triplicate and expressed as a mean. For low values range was 2% and for high values was up to 22%).

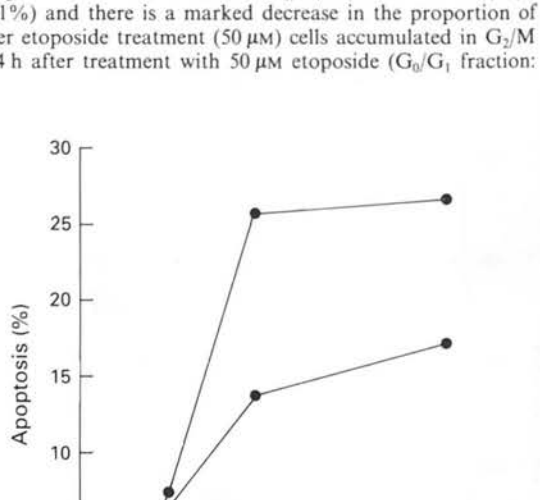

Figure 5 Clone 6 cells treated with 15 μU ml^{-1} bleomycin sulphate for 1 h at 37°C (●) and at 32°C (■). Note that treated cells incubated at 37°C undergo substantial apoptosis whereas treated cells at 32°C and untreated controls (open symbols) do not shown an increase in percentage apoptosis. Each line represents a separate experiment (performed in triplicate and expressed as a mean. For low values range was 2% and for high values range was up to 12%).

1994; Griffiths *et al.*, 1995) and myeloid progenitor cells (Lotem and Sachs, 1993) or in establishing a state of G_1 arrest, possibly permitting DNA repair (Lane, 1993; Bakalkin *et al.*, 1994). Clearly the cellular context in which p53 is expressed is important. Murine fibroblasts or primary rat kidney cells can be induced to undergo apoptosis by p53 in response to disruption of growth control by coexpression of c-*myc* (Wagner *et al.*, 1994) or adenovirus E1A (Debbas and White, 1993) respectively.

The finding that p53 function is lost in many authentic human and experimentally induced animal tumours has led to the assumption that p53 loss of function is causally associated with resistance to anti-cancer therapy (Lowe *et al.*, 1993*b*). In this study we have addressed the importance of p53 status on the sensitivity of cells to apoptosis induced by two anti-cancer drugs.

We have shown here, in a fibroblast cell line transformed with activated Ha-*ras* and temperature-sensitive p53 transgenes that wild-type p53 leads to G_1 arrest and at the same time resistance to the DNA damaging agents bleomycin and etoposide. By contrast, in the presence of mutant conformation p53, cells underwent apoptosis associated with a relative accumulation in G_2/M, a common response to DNA injury in yeast and mammalian cells (Hartwell and Weinert, 1989). We were unable to produce a G_0/G_1 arrest in Clone 6 cells at 37°C by either mimosine treatment or serum starvation as these treatments caused the death of the cultures. We were thus unable to show directly that a growth arrest in G_0/G_1, independent of p53, was protective against DNA damage.

Our findings apparently contrast with published work in which temperature-sensitive p53 was expressed in the M1 myeloid leukaemic (M1; Yonish-Rouach *et al.*,1993) and murine erythroleukaemic (MEL; Ryan *et al.*, 1993) cell lines induced apoptosis upon incubation at 32°C (i.e. with wild-type p53). MEL cells underwent G_1 arrest before undergoing apoptosis, but in M1 cells, no growth arrest could be observed at any position in the cell cycle. In addition, other cell types (including rat fibroblasts) have been shown to undergo G_1 arrest but not apoptosis in response to wild-type p53 induction (Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Kastan *et al.*, 1992). While bleomycin and etoposide maximally kill cells in S-phase, where replication forks are forced to negotiate either cleaved complex / double strand breaks (etoposide; Bae *et al.*, 1988) or double-strand breaks resulting from free-radical attack (bleomycin; Kuo, 1981), they can damage and kill cells in G_0/G_1 (Roy *et al.*, 1992; Clarke *et al.*, 1993; Evans *et al.*, 1994). In cell lines derived from clinically sensitive human tumours, DNA injury-induced wild-type p53 was held to be responsible for decreased clonogenicity following ionising radiation and this effect could be reversed by transfection of a dominant negative mutant p53. (McIlwrath *et al.*, 1994). The simplest explanation of *our* data is that the G_1 arrest mediated by p53 facilitates survival of *ras*-transformed fibroblasts by allowing effective DNA repair and prevents entry into S-phase, a stage when cells are often most susceptible to DNA damage.

Depending upon the cell system chosen, induction of p53 can cause either G_1 arrest, apoptosis or both apoptosis and G_1 arrest (Michalovitz *et al.*, 1990; Debbas and White, 1993; Ryan *et al.*, 1993; Yonish-Rouach *et al.*, 1993; Wu and Levine, 1994). The mechanisms by which decisions are taken that favour any of these end points are poorly defined but these decisions can be affected by specific growth factors (Yonish-Rouach *et al.*, 1991; Gottleib *et al.*, 1994; Canman *et al.*, 1995). In particular, it is not known how p53 can mediate apoptosis in the thymocyte but not in the fibroblast. The recognition of DNA damage (possibly involving the ataxia telangiectasia gene products; Kastan *et al.*, 1992) leads, via p53, to the control of the cell cycle at the G_1 checkpoint. We have shown this pathway to be protective in fibroblasts. Our results complement those of Lowe *et al.* (1993*b*) who showed that p53-normal fibroblasts were susceptible to anti-cancer treatment as a result of abrogation of the p53-mediated G_1 arrest by adenovirus EIA expression. Further, interleukin 6 (IL6) protects M1 cells from undergoing p53-mediated cell death (Yonish-Rouach *et al.*, 1991, 1993) and this protection also correlates with the induction of a G_0/G_1 arrest. (Levy *et al.*, 1993).

Wafl (Cip1 / sid1, p21), a gene product which is induced by wt p53, has potent inhibitory activity on cyclin E / cdk2 complexes in cells undergoing radiation-induced G_1 arrest (El-Deiry *et al.*, 1993, 1994; Dulic *et al.*, 1994). Waf1 is therefore a major regulator of cell cycle progression at the G_1/S interface. The expression of Waf1 in cell types that undergo apoptosis following activation of the p53 pathway suggests that it may be active in both arrest and death mechanisms. The decision of a cell to die may therefore be determined by other lineage-dependent messages or growth factors (Canman *et al.*, 1995), although the activity of *Waf1* as an apoptosis-inducing gene has not yet been directly tested. One such determinant may be the level of activity of the transcriptional regulator E2F-1. When constitutively overexpressed in the presence of wild-type p53 this triggers death in fibroblasts (Wu and Levine, 1994).

Using a different mutated p53 (proline substituted at residue 193) under its physiological promoter, Bristow *et al.* (1994) have recently shown that co-transfection of activated Ha-*ras* and mutated p53 into a primary rat embryonal fibroblast cell line resulted in enhanced clonogenicity *in vitro* and tumorigenicity in severe combined immunodeficient (SCID) mice after irradiation compared with cell lines containing *ras* alone. This effect was dependent on the level of mutant p53 expression, presumably as a result of competition with endogenous wild-type p53. However they did not directly assess the proportion of cells undergoing proliferation, growth arrest or cell death. We could not carry out experiments similar to those of Bristow *et al.* (1994) with ionising radiation sources as we found that reproducibility of results could not be maintained if there were fluctuations in temperature of Clone 6 cells before or during experiments. Indeed, clonogenicity of Clone 6 at 32°C is negligible.

Our *in vitro* experiments with DNA-damaging drugs (including the radiomimetic bleomycin) show that, under certain circumstances, overexpression of wild-type p53 can protect a cell which has suffered DNA injury against death rather than kill it, by causing cell growth to arrest in G_1. The corollary *in vivo* is that wild-type p53 in an appropriate cellular context could confer a state of increased drug resistance. The significance of mutated p53 oncosuppressor gene in clinical drug resistance is likely to be both complex and variable depending on the existence of other pathways of cell cycle activity control and response to injury. We show here that death of fibroblasts induced by etoposide and bleomycin occurs independently of wild-type p53 function. This confirms work by Strasser *et al.* (1994) which showed that thymic lymphoma cells from p53 – / – mice underwent apoptosis by p53-independent mechanisms following irradiation. Tumours which contain cells with mutated p53 initially may be more susceptible to cell death caused by therapy. However in the absence of G_1 arrest caused by wild-type p53, and therefore in the presence of continuing cell cycle activity, combined with karyotype instability (Livingstone *et al.*, 1992; Yin *et al.*, 1992), clones resistant to therapy may appear thus conferring a clinical state of 'drug-resistant' disease.

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Cell death in health and disease: the biology and regulation of apoptosis

Christopher O.C. Bellamy, Roger D.G. Malcomson, David J. Harrison and Andrew H. Wyllie

Apoptosis is a morphologically stereotyped form of cell death, prevalent in multicellular organisms, by which single cells are deleted from the midst of living tissues. Recognition of the cellular corpses and their removal by phagocytosis occurs without disturbance to tissue architecture or function and without initiating inflammation. Apoptosis is regulable and is of fundamental importance to tissue development and homeostasis. Cellular susceptibility to apoptosis is determined by a variety of signals, of both extracellular and internal origin, including proliferative status. Dysregulated apoptosis is important in the pathogenesis of several important human diseases including neoplasia, and recognition of the defects involved is prompting development of new therapeutic strategies.

Key words: apoptosis / genetic regulations / homeostasis

PHYSIOLOGICAL CELL DEATH is an inconspicuous yet prevalent phenomenon in complex multicellular organisms. It is characterized by the deletion of scattered, single cells from the midst of a living tissue without disturbance to the continuity of tissue architecture or function. With few exceptions cellular death is accomplished by a process with a structural stereotype, termed apoptosis, that strongly suggests a common underlying effector mechanism.[1] Apoptosis is observed in circumstances as diverse as embryogenesis, normal adult tissue turnover and organ atrophy; it is also fundamental to the regulation and operation of pathophysiological processes such as the immune response, inflammation and the elimination of cells after genotoxic injury (Figure 1). The realisations that apoptosis represents an innate cellular defence against carcinogenesis, that the regulatory pathways to apoptosis (but not the effector mechanisms) are frequently disabled in malignant neoplasms and that many cancer chemotherapeutic agents may act by induction of apoptosis have stimulated intense investigation into the underlying molecular controls.

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The structural changes of apoptosis

In apoptosis coordinated changes occur in the nucleus, the cytoplasm and at the cell surface (Figure 2).[2] The time to onset of apoptosis after a lethal stimulus is variable but the changes are rapid (a few minutes duration): cells lose contact with their neighbours and round up. The endoplasmic reticulum dilates and superficial cisternae fuse with the plasma membrane. Other cytoplasmic organelles remain largely unaffected. At the same time there is a striking loss of cell volume, apparently due to voiding of water and ions with consequent compaction of the organelles and an increase in cell density. The nucleus condenses and chromatin marginates to form dense granular caps under the intact nuclear membrane. The nucleolar fibrillar centre dissociates from its transcriptional complexes. The cell surface starts to bleb violently and time lapse phase contrast studies show an extraordinary bubbling appearance.[3] Around this time the nucleus breaks up into several membrane-bound fragments. The cell itself then splits into multiple membrane-bound 'apoptotic bodies', some of which contain nuclear fragments. The apoptotic bodies are phagocytosed almost immediately by neighbouring cells or by macrophages without eliciting an inflammatory reaction, in contrast to necrosis as described below. Apoptotic bodies in phagosomes remain recognizable by light microscopy for up to a few hours and are consequently the predominant form of apoptosis recognized in tissue sections. In epithelia or cultured monolayers apoptotic bodies can instead be shed into lumina or the culture medium where they degenerate within a couple of hours: they gradually lose membrane integrity (becoming permeable to vital dyes) and metabolic activity ceases.

Apoptosis is not the only possible mode of cell death. Necrosis is a non-specific term for a variety of

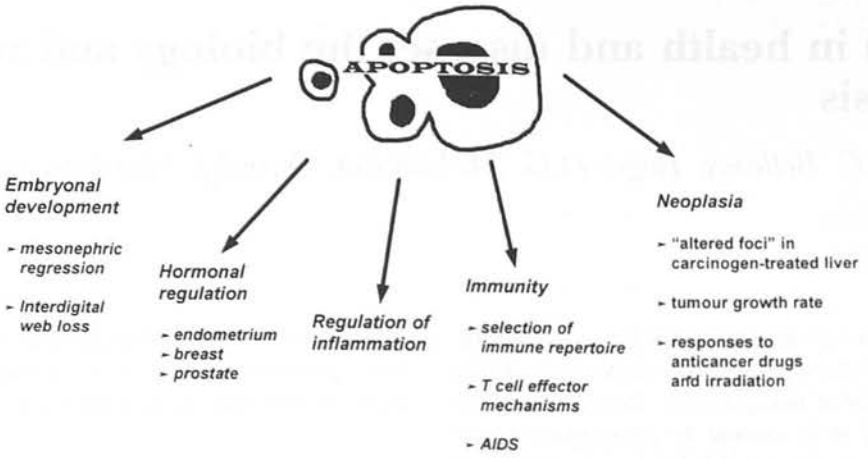

Figure 1. Prevalence of apoptosis in physiological and disease contexts (modified and reproduced with permission of John Wiley and Sons, Chichester, UK).

other modes of death in which, in contrast to apoptosis, the cell plays only a passive role while it is destroyed. Cell lysis during necrosis releases intracellular contents into the extracellular space, resulting in inflammation and further secondary tissue damage. Necrosis typically involves contiguous cells and is associated with tissue architectural disruption whilst apoptosis generally affects scattered single cells and preserves the tissue architecture (except in special circumstances such as embryonic remodelling).[1] Necrosis is always pathological whereas apoptosis is a physiological process that may also be triggered in pathological situations.

Kinetic considerations

The 'gold standard' for identification of apoptosis is

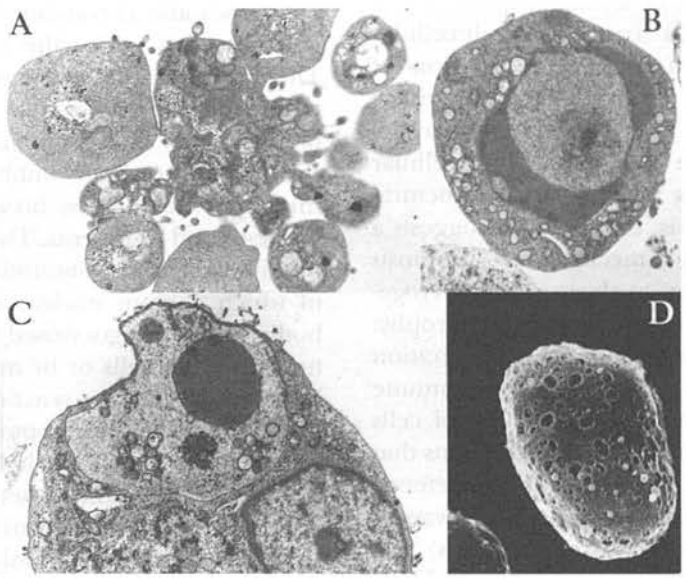

Figure 2. (A) Transmission electron micrograph showing late stage apoptotic fibroblast with fragmentation of the cell into membrane-bound apoptotic bodies. (B) Early apoptosis showing distinct peripheral chromosome condensation against the intact nuclear membrane. (C) Phagocytosed apoptotic body within a phagosome. (D) Cell surface changes in apoptosis: scanning electron micrograph of an apoptotic thymocyte, showing loss of microvilli and gaping cisternal pits, formed by fusion of dilated endoplasmic reticulum with the surface. (Reproduced with permission of Kluwer Academic Publishers, Dordtrecht).

morphological assessment. A comment on methods of evaluation of apoptosis has been given elsewhere[4] but the importance of quantitation is worth stressing. The speed of apoptosis and the rapidity of clearance of apoptotic bodies *in vivo* (a few hours at most)[5] mean that the identification of only a few apoptotic bodies in a tissue section can represent a considerable degree of cumulative cell loss. Numerically small differences in 'apoptotic indices' (i.e. the percentage of cells that are apoptotic) can therefore be of great biological import. For example, an intravenous bolus of anti-CD4 antibody increases the apoptotic index in murine lymph nodes from 0·06% to 1·33%, and this is sufficient to halve the total cell count of the lymph nodes within 48 h.[6] Unfortunately studies of this nature require that large numbers of cells be counted in order to achieve running means for apoptotic indices and allow statistical evaluation of differences. Furthermore, to be able to ascribe changes in tissue or tumour size to altered rates of apoptosis, parallel quantitative evaluation of cell proliferation is necessary. Other factors such as the rate of disposal of apoptotic bodies will also affect the perceived apoptotic index.

Underlying cell biology of the effector processes

Cytoplasmic events

The abrupt increase in **cell density** is due to voiding of water and ions, possibly channelled to the cell surface through the endoplasmic reticulum.[2] As yet no mechanism has been identified to account for this profound and sudden fluid shift. The cell size and shape changes during apoptosis require major **cytoskeletal reorganization** that is still largely uncharacterized, although actin polymerization (in part stimulated by protein kinase C) is essential for the budding that generates apoptotic bodies.[7] Some apoptotic cells, e.g. hepatocytes, also activate **tissue transglutaminase** to produce an insoluble shell of cross-linked protein.[8] Although increased transcription of specific mRNAs frequently precedes apoptosis (see below), a site-specific endogenous **RNAse** activity late in apoptosis is suggested by the rapid degradation of ribosomal and messenger RNA in apoptotic cells.[9,10] The role of **proteolysis** (e.g. of terminin proteins)[11] is unclear but specific regulatory proteases are consider later.

Nuclear events

As mentioned above, the nuclear membrane is not lost during apoptosis, unlike mitosis. However the subjacent **nuclear lamina** (which anchors chromatin) is dissassembled by depolymerization of constituent lamin filaments.[12,13] Lamin phosphorylation and depolymerization also occurs during mitosis, catalysed by cdc2 kinase, and it is possible the same mechanism operates during apoptosis.[14] That elements are common to mitosis and apoptosis is as likely to reflect a general role in regulation of nuclear structure as to indicate any deeper similarity between these distinct processes. In contrast to mitosis the depolymerized lamins subsequently undergo proteolysis in apoptosis, perhaps making lamina dissassembly irreversible.[13] **Ubiquitin conjugation** of nuclear proteins appears to be important in some (but not all) examples of apoptosis, and has been suggested to regulate some of the chromatin structural changes.[15] A specific feature of apoptosis is rapid **DNA cleavage**. Initially large transient 50 kbp and 300 kbp fragments are detectable that probably represent chromatin loops and rosettes detached from the anchoring nuclear matrix.[16] In many cell types there is further rapid and extensive double strand cleavage of internucleosomal DNA to yield a series of oligonucleosome chains of 180-200 bp multiples (180 bp is the length of DNA in a single nucleosome). These are visualised as the characteristic 'DNA ladder' on agarose gel electrophoresis.[2] The DNA cleavage is not sequence specific and the precise identity of the nuclease(s) responsible is still unknown (see ref 17, A. Eastman this issue, pp45-52, for a detailed discussion). It is sufficient to say here that nuclease is constitutively present in some cell types but is induced in others prior to apoptosis. The nuclear changes of apoptosis can occur without oligonucleosome generation (e.g. TGF β1-induced rat hepatocyte apoptosis), which when present may be a very late event, after chromatin margination.[16,18,19] Of note is that mitochondrial DNA is not fragmented in apoptosis, indicating that DNA fragmentation is a specific nuclear event.[20,21]

Surface changes

A key property of apoptotic bodies is their rapid recognition and phagocytosis by adjacent cells or professional phagocytes. Cell surface changes during apoptosis that promote recognition are best characterized for apoptotic inflammatory cells, and include loss of sialic acid (thereby exposing glycoprotein side-

chain sugars) and exposure of membrane phosphatidylserine.[22] A third mechanism involves thrombospondin secreted by macrophages to form a molecular bridge between apoptotic cell and macrophage surface CD36 or $\alpha_3\beta_3$ integrin.[22] Importantly phagocytosis of apoptotic cells does not activate macrophages to produce an inflammatory response.[1]

Organization of effector events

An ordered set of molecular events underlies the phenomena of apoptosis. To what extent these events are organized as a linear cascade or as parallel independent processes at multiple subcellular sites is unclear. It is of interest however that the nucleus is not essential for the cytoplasmic changes of apoptosis.[23] The degree of redundancy built into effector mechanisms is also unknown, although no obligate gene (i.e. without which apoptosis cannot occur at all) has yet been identified. **Expression of effector molecules** in non-apoptotic cells is variable: thymocytes must synthesize protein and RNA in order to undergo apoptosis in response to some stimuli,[24] but other cell types constitutively possess the effector molecules, as demonstrated by apoptosis in the presence of protein or RNA synthesis inhibitors. For other cells inhibition of protein synthesis itself triggers apoptosis, suggesting the presence of a short-lived protein inhibitor of cell death. These observations have led to the concept of states of readiness ('priming') for apoptosis, determined by the dynamic activity of regulatory pathways to induce or deplete effector molecules.[2] The idea has implications for chemotherapy in that cells in a highly primed state (with all the apoptotic machinery in place) might be more susceptible than unprimed companions to the triggering of apoptosis by cytotoxic stimuli. The increased sensitivity to cytotoxic agents of cells in 'proliferative' compartments of tissues when compared with non-proliferative compartments has been cited as an example of priming, although whether this difference is due simply to accumulation of effector molecules is unproven. In fact current models suggest that cell cycle activation is actually lethal itself, unless specific rescue factors prevail (see next section).

The regulation of apoptosis

Broadly speaking, physiological apoptosis can be externally triggered or a cell-autonomous event (Figure 3). **Cell-autonomous apoptosis** is a hard-wired phenomenon, often termed 'programmed cell death'. It is exemplified in morphogenesis by the coordinated death of web space cells that sculpts digits from the coarsely-shaped limb bud,[1] and by the chronologically and spatially invariant death of 131 cells (12% of total, excluding germ cells) that occurs during development of the nematode *Caenorhabditis elegans*.[25] The nature of the internal clock or switch that activates death is not known, although study of mutant *C elegans* with abnormal cell death phenotypes has led to characterisation of some of the genes involved, as

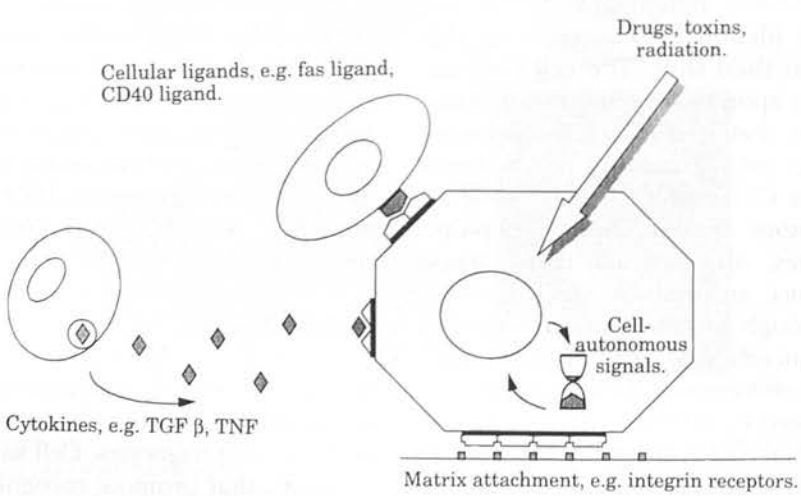

Figure 3. Cartoon illustrating the diverse sources of signals that regulate susceptibility to apoptosis.

discussed below. The rationale for this sort of inevitable cell death is easy to appreciate in limb morphogenesis but is less clear for *C elegans*, where mutant animals that lack programmed cell deaths survive and appear grossly normal. Observed minor behavioural changes and slower maturation have been hypothesized to put the cell death-deficient animals at a selective disadvantage compared with wild-type.[25]

External signals

Specific ligand-receptor binding can transduce both death and survival signals. It is of relevance that a 'survival factor' need not be a mitogen and also that mitogens are not necessarily survival factors; indeed there appears to be benefit in separating the two functions. The TNF receptor superfamily includes several members that participate in positive and negative regulation of cell death, for example TNFRI, CD40, NGFR, CD30 and the fas antigen.[26-28] Receptor activation is not necessarily determinate of outcome and *signal context* is critical. For example, TNFα-induced apoptosis is blocked by expression of the zinc finger peptide *A20*[29], and fas stimulation kills chronically but not recently activated T cells, (a distinction postulated as a mechanism for limiting the extent of normal immune responses).[30] The importance of context is well illustrated by the dual signal model of B cell responses to crosslinking of surface immunoglobulin by antigen.[27] Crosslinking induces apoptosis, but not if a second signal is provided by costimulation of surface CD40. The ligand for CD40 is present on activated (but not resting) T helper cells, so apoptosis is only blocked during an antigen-specific immune response. Therefore, according to the model, self-reactive B cells will normally be deleted on encountering antigen, as a result of the absence of activated self-reactive T cells that could provide a rescuing signal. Thus the humoral signal of antigen binding is read in the context of a cellular signal (CD40 binding). Some agents have **dual effects** on the same cell population, for example TGFβ1 is a cytokine that both inhibits DNA synthesis and stimulates apoptosis of hepatocytes and endometrial stromal cells.[31,32] It is not clear what determines the particular response at individual cell level.

Competitive selection

When apoptosis is the default fate of newly generated cells, competitive selection for survival factors is a powerful strategy to ensure that only those cells survive that are best suited to perform a particular function. Evidence from central nervous and haemopoietic systems suggests there are specific factors for different cell types and 'windows of susceptibility' during which cells are dependent upon particular factors.[33-35] This type of scenario is in contrast to the fixed, programmed cell death of *C. elegans*. For example, in neuromuscular development lower motor neurones are generated in excess and deleted by apoptosis if their axons fail to contact muscle end plates, the source of neurotrophic survival or rescue factors.[34] Likewise, during nephrogenesis, metanephric mesenchyme converts to epithelium under the influence of an inductive signal from the ureteric bud.[36] This signal rescues from the apoptosis that is the fate of uninduced cells. A similar principle brings about the increase in affinity of specific antibody produced during a humoral immune response (affinity maturation). This is achieved by selection for long term survival of only a small subpopulation of centroblasts, based upon the relative affinity of their surface immunoglobulin for the antigen, presented on follicular dendritic cells.[37] The remainder die by apoptosis in the light zone of the lymph node germinal centre, being seen as 'tingible bodies' within adjacent macrophages. Accessory survival signals are provided by cell adhesion molecules on the follicular dendritic cells, ICAM-1 and VCAM-1, which bind centroblast LFA-1 and CD49d respectively.[38]

Dependence on survival factors is also a key method of cell **population size regulation**. In kinetic terms a continuously renewing tissue (e.g. gut epithelium, bone marrow) consists of stem cells, progeny transit cells (which may or may not divide a few times) and post-mitotic, differentiated cells. There is normally overproduction of transit cells of which only a proportion survive to maturity, through the action of specific rescue factors, e.g. IL-1α, IL-6 for myeloid progenitor cells. The concentration of transit cell rescue factors is thus limiting and a determinant of final population size. Paracrine survival factors also maintain tissue localisation, e.g. within bone marrow. A requirement for survival factors by mature cells, which may differ qualitatively from that of immature cells, is illustrated by the atrophy of thyroid or adrenal glands after hypophysectomy, prostatic regression after castration and involution of post-lactational breast parenchyma that are all characterised by increased apoptosis.[1,2] The social control hypothesis provocatively takes the concept of survival factors to

its limit by suggesting that *all* cells (except blastomeres) are *continuously* dependent on survival signals from other cells to avert an intrinsic death program.[35]

This section has so far described how soluble factors and cell-cell interactions can control cell death, but cell-matrix interactions also regulate apoptosis. Specific contact of surface integrin receptors with extracellular matrix molecules is an important survival signal for differentiated endothelial and epithelial cells.[39,40] Without integrin-matrix binding these cells undergo apoptosis, a phenomenon that has been termed anoikis ('homelessness').[40] Such cells cannot therefore survive out of position if the appropriate matrix requirements are not fulfilled. Thus matrix composition localizes seed to soil in an unforgiving manner, a concept of critical importance to understanding mechanisms of neoplastic progression. In contrast, fibroblasts do not show integrin dependence,[40] in keeping with the need to rove across tissue boundaries during repair of injury.

Signal transduction

The signal generated by a stimulus must be transmitted to effector molecules that often lie in different subcellular compartments. Transduction pathways vary according to the trigger stimulus but two major areas of focus are cytosolic calcium and protein kinases. Apoptosis is usually preceeded by a rise in cytosolic calcium concentration and possible downstream targets include calmodulin and calpain.[41,42] Calmodulin and calpain are themselves pleiotropic molecules whose precise roles in apoptosis need to be clarified. Protein kinase C (PKC) has been identified as both a positive and negative regulator of apoptosis,[42] while a protein kinase A pathway is active in thymocyte apoptosis.[43] It is possible, though without proof, that the conflicting roles of the PKC family may reflect the selective involvement of PKC isotypes[44] or interaction with other signalling pathways such as sphingomyelin-ceramide.[45] The *ras* pathway has long been known to transduce growth signals from plasma membrane to nucleus but is also a negative regulator of apoptosis when overexpressed in fibroblast cell lines.[46,47] Overexpression of activated *raf* kinase, a downstream element in the *ras* pathway, was also found to prevent apoptosis induced by IL-3 withdrawal.[48] New evidence for crosstalk between these different pathways is revealing a complex network of vertical and horizontal interactions that integrates different signals to determine the outcome of a stimulus.[49] The machinery for context-dependent responses is thus coming to light. A major unanswered question is how these pathways converge on the common effector processes of apoptosis.

Genetic regulation of apoptosis: strategies for investigation

Simple observational studies on tissues can be very informative, providing physiological paradigms for cell death. These studies indicate the tissue compartments most susceptible to apoptosis under basal conditions, and can also provide prima facie evidence for a role for apoptosis in specific disease.[2,50] In *C. elegans* and *Drosophila*, observation of abnormal cell death phenotypes in mutant animals has allowed genetic analysis to identify novel genes controlling cell death.[25,51,52] The characterization of mammalian homologues of such genes and the use of double mutant animals to order the genes in a 'death pathway' show the power of the model.[25,53,54] Conversely, specific genetic manipulation of animals or cells offers clues to the functions of known genes of interest. Antisense aligonucleotides are a potentially powerful tool to abrogate a particular gene's function, although in practice a number of confounding factors present themselves. In fact each strategy inevitably has significant advantages and disadvantages: for example, drugs are powerful tools for the study of apoptosis; many are cancer chemotherapeutic drugs and as they reveal more about the regulation of cell death so that information in turn will suggest novel strategies of chemotherapy (see ref 55, this issue). However, interpretation of the effects of pharmacological agents must discriminate action at specific points on physiological death pathways from non-specific activation of a suicide program. Likewise, genetic experiments that use the unregulated overexpression of genes in (genetically abnormal) cell lines are susceptible to criticisms of physiological relevance. Animals with germline targeted knockout of selected genes are a more physiological model, yet unique gene functions will still be much more readily apparent than those less critical or redundant, but which have relevance. Transgenic animals carrying extra genes in the germline that are constitutively overexpressed are also powerful models but still difficult to interpret in terms of normal function.

Together these different approaches have provided information that forms a surprisingly concordant picture of the internal regulation of apoptosis. They have delineated genes of three broad classes in a

common mechanism — those suppressing apoptosis, those defining the final common activation elements and those 'upstream' of the suppressors, but 'downstream' of the signal transduction described earlier, which we call here intermediate genes.

Genes suppressing apoptosis

Induction of the *bcl-2* gene is often critical in the action of survival factors. *Bcl-2* is a mammalian homologue of the *C. elegans ced-9* gene, whose function it can partially replace,[53] and it can prevent apoptosis caused by a variety of physiological, pathological and pharmacological stimuli.[56] How *bcl-2* works is uncertain, although presumably it acts close to the final irreversible steps of apoptosis on which afferent pathways converge. There is evidence that *bcl-2* affects calcium partitioning and cellular redox status, although this is still controversial (see ref 57, RW Craig, this issue, pp35-43, also 58,59). However *Bcl-2* is not a universal antidote to cell death. For example, ectopic *bcl-2* expression by cortical thymocytes can prevent the induction of apoptosis by irradiation, glucocorticoids and antibodies to CD3, but does not affect negative selection.[60] This may be partly due to the presence of *bcl-2* antagonists such as bcl_{XS} which is expressed at high levels in immature ($CD4^+CD8^+$) thymocytes.[61] Bcl_{XS} has not been shown to interact directly with *bcl-2* but another antagonist, *bax*, seems to exist in dynamic equilibrium between *bax-bax* homodimers that are permissive for apoptosis, and *bax-bcl-2* heterodimers which may be a biologically active form of *bcl-2* that suppresses apoptosis.[62,63] It is not yet clear whether the *bcl-2* antagonists simply quench survival functions of *bcl-2* or whether they have intrinsic lethal properties that *bcl-2* must hold in check. In either case the relative amounts of such molecules in a cell could predetermine its response to a lethal stimulus. In tissues where *bcl-2* is not essential for survival such as the CNS (which develops normally in mice lacking *bcl-2*),[64] functional analogues exist, such as bcl_{XL} which is expressed at high level in brain.[61] The antiapoptotic actions of the *ras* pathway were discussed earlier and it will be of interest to determine whether the *bcl-2* family interacts with *ras/raf* transduction pathways. *Abl* is another antiapoptotic gene that in contrast to *bcl-2* is biochemically well defined. The *abl* product is a tyrosine protein kinase and a constitutively activated form, retroviral *v-abl*, can suppress the apoptosis that follows growth factor withdrawal *in vitro*.[65] Protein kinase C and phospholipid hydrolysis may be mediators of this effect.[66] An *abl* fusion product (*bcr-abl*) in Philadelphia chromosome-positive human leukaemic cells is also constitutively activated and probably suppresses apoptosis in the malignant cells.[67] In addition to *v-abl* viruses have evolved a number of strategies to suppress apoptosis and these are discussed separately below.

Distal activating genes

The *ced3* and *ced4* genes of *C. elegans* are strong candidates for distal activating genes if not ultimate effectors of apoptosis.[25] One vertebrate structural homologue of *ced3* is ICE (interleukin-1β converting enzyme), a cysteine protease that when overexpressed in fibroblasts induces apoptosis and inhibitors of which prevent the neuronal apoptosis that follows NGF withdrawal.[54] The universality of ICE's ability to induce death and its actual relevance to physiological apoptosis of normal cells are yet to be tested and it is likely that a family of ICE-related proteases will be identified. In some systems *bcl-2* is able to inhibit ICE-induced cell death, placing it downstream of ICE-like proteases in regulation of apoptosis.[54] Analogous attempts to order the *ced3/ced4* and *ced9* genes in a *C. elegans* putative death pathway are more speculative. Animals with *ced9* gain of function do not show *ced3/ced4*-dependent cell deaths, whilst animals lacking *ced9* function show excessive and abnormal *ced3/ced4*-dependent cell deaths.[52] If the three genes lie along one linear pathway then these observations place *ced9* proximal to *ced3/ced4*.[52] However, if *ced9* activation is a separate pathway to a *ced3/ced4* death cascade then *ced9* action could be either proximal or distal to *ced3/ced4* and still be consistent with the data above. Given the many similarities of *ced9* to *bcl-2* and of *ced3* to ICE it would seem reasonable at present to expect *ced9* to act downstream of *ced3*. The reaper peptide of *Drosophila* also seems to be a distal activator for apoptosis.[51] *Reaper* expression precedes apoptosis by 1-2 hours and is detectable in apoptotic bodies. Deficiency of *reaper* blocks developmental and radiation-induced apoptosis, although not completely, showing that *reaper* is not obligate for apoptosis to occur. Reaper has no homology to known peptides and the effect of expression in vertebrate cells is not yet reported.

Intermediate genes

Many genes that regulate apoptosis were first identified as oncogenes or oncosuppressor genes. The c-*myc* oncogene is classically associated with promotion of

cell division and one might expect that constitutive activation of *myc* would be sufficient to induce autonomous cell proliferation. However, this is not so; in culture, activation of *myc* induces apoptosis unless specific rescue factors are also present, for example IGF-1 for fibroblasts or IL-3 for myeloid cells.[3,68] Thus *myc* activation simultaneously generates two possible outcomes in these cells: death (by default) or proliferation (if rescued) (Figure 4). Deregulated expression of the *myb* oncogene, at least in myeloid cells, may have a similar effect.[69] Such coupling of oncogene activation to survival factor dependence may serve two purposes. It opens a window of susceptibility to apoptosis (a 'high turnover' state), allowing competitive selection of susceptible cells to regulate population size as described above. It also forms an inbuilt safety mechanism to delete cells in the event of incongruous oncogene activity. A corollary of the 'high turnover' state model is that because proliferating cells in general are poised for apoptosis they will be susceptible to a variety of triggering agents, including cancer chemotherapeutic agents. In support of this idea epithelial cells in the proliferative zone of gastrointestinal crypts are more sensitive to irradiation and cytotoxic agents than adjacent non-proliferating crypt cells.[70] Tissue compartments in a high turnover state are therefore in a precarious balance between population expansion (excess proliferation) and regression (excess apoptosis), determined by the availability of mitogens, survival factors, cytotoxic stimuli and recruitment into or out of the high turnover state, e.g. differentiation. At a molecular level it is unclear whether the coupling of cell cycle activation to apoptosis is specifically mediated by *myc* or by a downstream component of an active cell cycle.

The *p53* oncosuppressor gene is the most frequently mutated gene in human malignancy (see ref. 71, C.E. Canman, M.B. Kastan, this issue, pp17-25). It is critical for a DNA damage response to DNA strand breaks, produced either directly or during the excision-repair of other DNA lesions. In different cell types, such breaks result in G1 arrest or apoptosis.[72,73] What determines the differences in response is unclear, although of interest is the observation that overexpression in a cell line of transcription factor E2F-1 (a positive regulator of *myc* expression[74] involved in cell cycle regulation), switched a *p53*-dependent G1 arrest to a high turnover state with excess apoptosis.[75] E2F is shut off by hypophosphorylated pRb (the active retinoblastoma gene product), a negative regulator of cell cycle progression that is inactivated by cyclin-dependent kinases (cdk) in G1, themselves a target in *p53*-induced G1 arrest (via WAF1).[76-78] An interesting pathway is thus sketched out, in which *p53*/WAF1 growth arrest is achieved through cdk inhibition to activate pRb, which then shuts off E2F-1. However defects such as loss of pRb

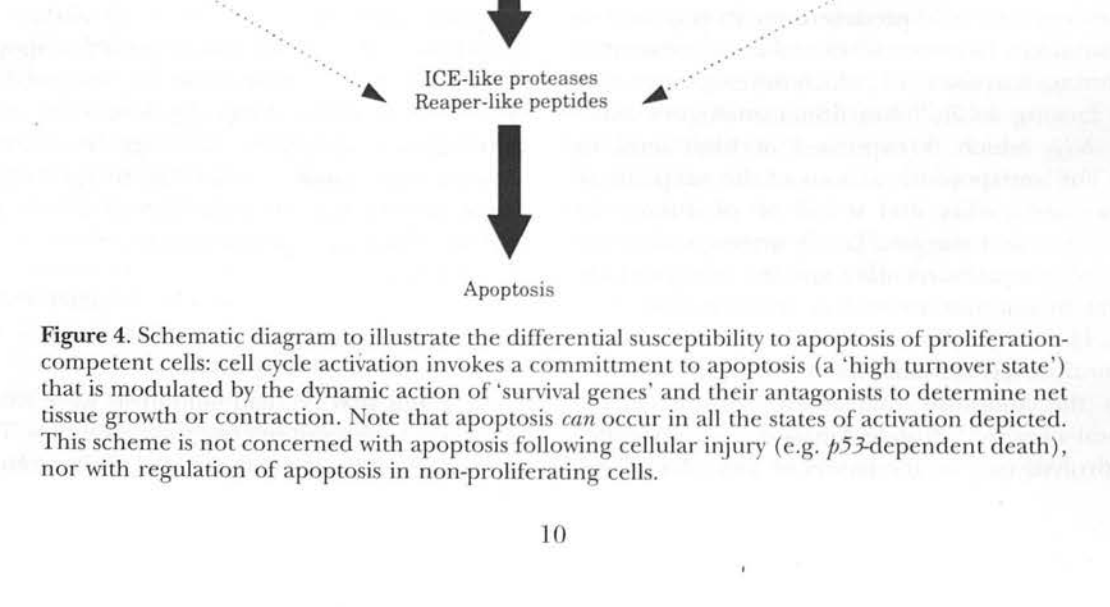

Figure 4. Schematic diagram to illustrate the differential susceptibility to apoptosis of proliferation-competent cells: cell cycle activation invokes a committment to apoptosis (a 'high turnover state') that is modulated by the dynamic action of 'survival genes' and their antagonists to determine net tissue growth or contraction. Note that apoptosis *can* occur in all the states of activation depicted. This scheme is not concerned with apoptosis following cellular injury (e.g. *p53*-dependent death), nor with regulation of apoptosis in non-proliferating cells.

function would prevent shut-down of E2F-1, which could then induce a high turnover state, as described above. This model is supported by the example of mice deficient in the retinoblastoma gene product. They die *in utero* with CNS and haemopoietic abnormalities, characterized by excessive proliferation and apoptosis.[79] The direct mechanisms of *p53*-dependent apoptosis are also becoming clearer. p53 downregulates *bcl-2* expression and also upregulates its antagonist *bax*.[80,81] This suppresses a major anti-apoptotic pathway and places *p53* proximal to *bcl-2/bax* in the regulation of apoptosis.[82]

The purpose of the decision to enter G1 arrest after DNA damage is unclear but one attractive hypothesis suggests that it provides an opportunity to repair DNA lesions before DNA replication occurs.[83] If the damage is in some way recognized as irreparable, the hypothesis suggests that *p53*-dependent apoptosis would be triggered, to prevent the replication of damaged DNA and a gradual accumulation of genetic defects that might result in carcinogenesis. In support of this hypothesis thymocytes and gastrointestinal crypt cells of p53-deficient mice lack the normal apoptotic response to DNA damaging agents, intriguingly in a gene-dose dependent fashion.[84,85] In addition *p53* has been shown to be critical for the maintenance of genomic integrity in serially passaged cells, and mice without functional *p53* genes die prematurely from malignancies.[86,87] Thus *p53* is involved in the policing, the ministration and the execution of cells with DNA damage. It is hardly surprising therefore that *p53* mutations are common in malignancy, given the advantages for neoplastic progression that disabling of *p53* confers.

p53 is not critical for all apoptotic pathways (for example murine thymocytes from *p53*-deficient animals retain a normal apoptotic response to glucocorticoid),[84] but it is implicated in apoptosis effected *in vitro* by mechanisms apparently unrelated to DNA damage. Thus *p53* is required for normal sensitivity *in vitro* of haemopoietic cells to survival factors and loss of even a single allele reduces (but does not remove) the requirement for survival factors.[88] Introduction of wild-type *p53* into a leukaemic cell line has also been reported to generate dependence on IL-6 for survival, and p53 was necessary for the apoptotic response to serum withdrawal in a cell line cotransfected with adenovirus E1A and *ras* oncogenes.[89-91] Therefore, although mice deficient in *p53* develop normally[84] and *p53* is clearly not essential for developmental cell death, altered survival factor thresholds may influence population selection during development and provide a more favourable environment for carcinogenesis.

A number of other gene products are implicated in the control of apoptosis (e.g. clusterin, *c-rel*, *fos*, cyclin D1[92-95]), but in most instances it still remains to sort primary regulators from secondary perturbations.[96,97] Recent evidence from a myeloid leukaemia cell line has implicated the *Myd118* gene as a downstream mediator of TGFβ-induced apoptosis and has suggested that *bcl-2* inhibits apoptosis of these cells via downregulation of *Myd118* expression.[69]

Disease connotations: apoptosis in disease and the responses to disease

It is evident that apoptosis provides a powerful regulatory mechanism for many aspects of normal tissue growth and function. This section extends the discussion to describe how apoptosis regulates the responses to disease and how defective regulation of apoptosis may be central to the pathogenesis of many important disorders.

Inflammation

The response to injury or infection has itself considerable potential to damage tissue and it is therefore tightly regulated. Neutrophils, eosinophils and monocytes die by apoptosis within a relatively short period (e.g. 3-4 days for eosinophils in culture), however death can be significantly delayed by proinflammatory cytokines such as C5a (neutrophils), IL-1β, TNF-α, IFN-γ (monocytes) and IL-5 (eosinophils).[98,99] In contrast TGFβ and TNFα accelerate eosinophil and neutrophil apoptosis respectively.[100,101] This suggests a potential mechanism *in vivo* for control of the survival and ultimately the removal of these potentially dangerous cells from sites of inflammation when the inflammatory stimulus subsides. Defects in these mechanisms or in the clearance of apoptotic cells may underlie some chronic inflammatory diseases (e.g. hypereosinophilic syndromes) due to inappropriate persistence of inflammatory cells with continued release of toxic cellular contents perpetuating tissue injury and inflammation. Of interest in this regard is the multifocal inflammatory disease and tissue necrosis that occurs in mice without functional TGFβ1.[102]

Autoimmunity

Apoptosis is critical for the development, function and regulation of the immune system (see ref 103, B.A. Osborne, this issue, pp27-33). One instructive example is thymocyte maturation. A competent immune system must recognise a wide variety of foreign antigens but provision must be made to delete self-reactive effector cells that would otherwise cause autoimmune disease. In the thymus, 97% of thymocytes are deleted in their first few days of life.[104] This occurs as a result of a complex weeding-out process in which immature thymocytes die by apoptosis unless able to recognise and bind antigen presented in association with self MHC molecules (*positive selection* for MHC restriction) and yet die also if the receptor occupancy is too great (*negative selection*).[105] The principle is that high receptor occupancy is likely to reflect reactivity to self-antigen since most antigen presented in the thymus is self-derived. However, negative selection is certainly more complex than that, and further contributory mechanisms involving other surface signals are likely.[105-108] In concert with thymic selection, peripheral deletion of mature T cells is also important to prevent autoimmune tissue damage. One mechanism involves apoptosis induced by activation of surface fas molecules. Fas (CD95, Apo-1 receptor) is a member of the TNF receptor superfamily and like some other members can transduce a signal for either apoptosis or proliferation. Mice without functional fas or fas ligand accumulate abnormal $CD4^-CD8^-$ T cells and develop autoimmune disease resembling human systemic lupus erythematosus (SLE).[94] These mice have lost the antigen-driven fas-dependent apoptosis of mature T cells that maintains tolerance to self.[110] Further compelling evidence for a direct pathogenetic role of fas-mediated cell death is provided by the recent observations in SLE patients of elevated serum levels of a soluble form of fas at concentrations that blocked induction of fas-mediated apoptosis *in vitro*, and that in mice caused altered lymphocyte development and proliferation responses to self antigen.[111]

Cytotoxic lymphocyte (CTL) killing

Cell-mediated cytotoxicity is an integral component of specific host defences, for example against virally infected cells, and apoptosis is believed to be the mode of death in a proportion of CTL-induced target cell killing.[26] Evidence suggests that activation of target cell fas by the CTL is an important mechanism for this cell-mediated apoptosis, although engagement of target cell TNF receptors may also act to trigger apoptosis in some instances.[112,113] The finding that activation of surface fas on hepatocytes triggers apoptosis[114] suggests a potential pathogenetic mechanism for viral or perhaps autoimmune hepatitis that may have implications for new strategies of therapy. It is of relevance to note that CTL can kill by a mechanism that involves perforin insertion into target cell membranes and granule exocytosis.[26] The relative importance and interactions of these different modes of killing is not established.

AIDS

The gradual depletion of $CD4^+$ T cells during HIV infection that leads to clinical AIDS is thought to be due to excessive apoptosis.[114] HIV-infected cells express a viral envelope transmembrane gp120-gp41 complex which binds the CD4 D1 domain of uninfected T cells and triggers apoptosis directly.[115] Furthermore HIV particles shed gp120 which, although unable to trigger apoptosis itself, can bind CD4 and program uninfected T cells for apoptosis (instead of proliferation) in response to subsequent T cell receptor stimulation by antigen.[116] The deletion of naive and memory T cell clones on encountering their specific antigen abolishes the individual's ability to mount a specific immune response to infections.[6,116]

Neoplasia

Escape by cells from normal activation of apoptosis allows survival of 'forbidden clones' that would otherwise have been deleted. This might allow the propagation of, for example, new genetic defects that would have been eliminated through p53-dependent apoptosis. In addition, reduced dependence on survival factors may be important in the early stages of carcinogenesis to allow expansion of subpopulations of cells capable of subsequent progression to malignancy. An example is experimental liver tumour promotors such as nafenopin and phenobarbital that reversibly inhibit apoptosis with consequent hyperplasia and development of preneoplastic foci.[117] Constitutive bcl-2 expression by follicular lymphomas is an example of a death suppression strategy in human neoplasia.[118] The qualities that allow survival of malignant cells in foreign tissues have received little attention but are a *sine qua non* for invasion and

metastasis. It is likely that escape from integrin dependence is one such necessary event.

Oncogenic viruses

Oncogenic viruses have developed strategies to prevent host cell apoptosis that have shed light on control pathways. The Epstein Barr virus BHRFI protein is a *bcl-2* homologue, whilst the LMP-1 protein upregulates *bcl-2* expression and induces the *A20* zinc finger protein that confers resistance to TNFα cytotoxicity.[119-121] The adenovirus E1B gene encodes a functional homologue of *bcl-2* and a protein that inactivates the *p53* oncosuppressor.[122,123] In fact several viruses inhibit *p53* function in different ways, including SV40 (large T antigen), Epstein Barr virus (EBNA 5), human papillomavirus types 16 and 18 (E6 protein) and hepatitis B virus (HBx protein) (see ref 124 for references). This is a testament to the importance of that molecule in countering abnormal cell proliferation. Interestingly, many oncogenic viruses contain genes that activate cells from the growth arrested state (SV40 T antigen, adenovirus E1A, HPV E7) probably via inactivation of Rb protein, release of transcription factor E2F and activation of c-*myc*. At least some of these changes also imply increased susceptibility to apoptosis, as discussed earlier. The combination therefore of pro-apoptotic oncogenes with others having anti-apoptotic activity appears to be an essential part of the viral strategy to induce cell proliferation without also activating cell death.

Cancer therapy implications

The ability to modify sensitivity to apoptosis through the regulatory pathways has clear implications for the treatment of malignancy.[125] Potential strategies fall into three categories — direct triggering of apoptosis by cytotoxic agents, enhancing susceptibility to apoptosis to increase the efficacy of other therapies, and boosting the resistance of normal cells to apoptosis (with survival factors). Restoration of function of interrupted apoptotic pathways, e.g. *p53*-dependent apoptosis, with consequent self-deletion by tumour cells would be a most attractive strategy. Bcl-2 antagonists might likewise be expected to cause regression of follicular lymphomas or at least to increase their radio- or chemosensitivity. Induction of a high turnover state (with survival factor dependence) or antagonism of tumour survival factors (e.g. antiandrogens for prostate carcinoma, tamoxifen for oestrogen receptor-expressing breast carcinomas) are other approaches to therapy. Boosting normal cell resistance to apoptosis with exogenous survival factors can be used after ablative therapy to improve restoration of the normal cell population,[126] reducing treatment morbidity and allowing greater frequency of cytotoxic treatments.

Conclusion

Apoptosis has an importance in physiology and pathology that has only recently become fully appreciated. Its purpose is to rapidly delete single cells from living tissue without interrupting tissue function or structural integrity. Diverse fields of developmental, cell and molecular biology are linking to contribute to a deeper understanding of apoptosis that has a direct relevance to a variety of human diseases and in some instances suggests new therapeutic strategies. Much of the basic biology of the regulation and effector events of apoptosis remains obscure but the field is now subject to such an intense level of investigation that it is not unrealistic to expect great advances within a very short time.

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